

Safeguarding British research

Recent improvements of the climate in British research do not imply that the dangers have passed. Indeed, the most serious — complacency — is more serious now than ever.

SURPRISING as it may seem, the morale of the British research enterprise may be on the mend, if only slowly. There are several explanations, not the least of which is that the spate of bad news has abated to a mere trickle. Laboratories are still being closed — a meat research station a few weeks ago, now a couple of veterinary schools (see page 294) — but to those still with posts, the prospect that most institutions would be shrunken images of their present, let alone their past, selves seems to have receded. And there is to be some extra cash. In the financial year that begins in April, the government's direct subvention of basic research will increase by £95 million to a total of £850 million. Indeed, potential recipients of the extra funds might by now have known whether anything extra is likely to come their way if the government's announcement of how the total budget will be allocated between the research councils and other spending agencies, originally planned for earlier this week, had not unaccountably been postponed.

In the circumstances, there is a danger that alarm, itself begotten of complacency, will breed a further round of indifference. At a time when even British high-energy physics has been told (in last week's corporate plan of the Science and Engineering Research Council — see page 296) that there is no "wish to reduce the provisions for nuclear physics further", it may be all too tempting to suppose that the passage of time will mend the damage done to British research in the past decade, in due course even providing a platform for resurgence. But in reality, passivity of that kind will invite only further trouble. If there is now to be a breathing space, the research community should use it as an opportunity for healing wounds and for ensuring that the privations of the past decade will not recur.

It is important, first, to understand what has happened. The mere fact that laboratories have been closed and their members dispersed or put out to grass is in one sense water under the bridge. The process of contraction may have seemed arbitrary at the time, and unjust to many of the people who lost their jobs, but nobody would now recreate the same laboratories even if practical problems since brought to light cannot now as easily be solved. (The agriculture and food ministry's mishandling of the recent and still-current scare about the infection of eggs by *Salmonella* bacteria might have been avoided if the once-luxuriant agricultural research establishment were still in being.) Similarly, while British researchers may rightly regret the numbers of erstwhile colleagues who have gone for good elsewhere, there is probably no way now of bringing them back.

Damage

The much more serious and lasting damage is that done in people's minds. And even if researchers may now believe that there are smooth waters ahead, the minds of others will retain the impression of a research profession under threat for a long time to come. That this must be so is shown by the still laggard recruitment of young people to science courses at universities and polytechnics, and by the shortage of graduates clamouring for careers in research.

That is why the British research enterprise must from now on pay more attention to its public reputation, especially among

young people. Talking (and writing) about the excitements of research will help. So too will schemes such as those growing up between British universities and polytechnics with neighbouring groups of industries and schools. Although primarily intended to take the edge off recruitment problems, such devices may serve the broader purpose of demonstrating at first hand that research remains self-confident. But in the long run, what will matter more is that the research profession should be recognized as a successful one, either the matrix from which successful entrepreneurs spring or as one that enjoys the esteem of comparable professions, which means that among other things its members should be reasonably well-paid. There are some who remark regretfully that much of the government's extra £95 million this year will go on pay increases, but is that an unmitigated misfortune? Indeed, with the prospect of a true European Common Market no further away than 1992, should not the managers of the enterprise be planning to pay British scientists mainland salaries by then?

A further change that should not be forgotten is that worked by the pressures of the past decade on the temper of the grant-making agencies in Britain. The importance of the change is evident in the corporate plan (a name which is itself a pointer) of the Science and Engineering Research Council (SERC). Not so long ago, the council would have insisted in such a document that its function is to support research at universities; the range of laboratories it manages were all originally created to provide services (mostly equipment) that universities and polytechnics could not manage on their own. But SERC tells how, "in association with HEIs" (presumably an acronym for Higher Education Institutions), it will "continue ... to support excellence in ... science and engineering". Its declared aim is that the ratio of resources spent on "strategic" and "basic" research will be in the ratio 60:40, identifying the former with "wealth-creation" and letting the reader guess that the latter has no economic significance, except perhaps in giving young people skills that may be put to economic use.

The shift of emphasis from the support of research institutions to the support of research itself is significant, and worrying. SERC's own spending on research grants has increased in the past few years commendably, but what has happened to the old notion that researchers, not committees, are the best (and most imaginative judges) of what might be done next? The distinction between strategic and basic research, like all attempts meaningfully to partition a spectrum by drawing a line across it, similarly makes no sense. As the whole world knows, it is not possible to tell with any certainty which research projects will create wealth and which will be largely academic in their consequences. But if there is some substantial parcel of British research which is certain to produce wealth, it would do so more even more effectively if it were market-orientated. In other words, SERC's policy is an invitation to some future government to complain that 60 per cent of what it does would be better hived off to industry. By insisting that these simple judgements of SERC's are heresy of a kind, the scientific community in Britain will not merely assure the integrity and thus safety of research, but may even help to save SERC from itself. □



Soviet nuclear dissent

Chernobyl is not the only reason why Soviet civil nuclear power is behind target. Can it pull back?

With the possible exception of the government of France, the Soviet government has so far been among the most resolute (and successful) in the pursuit of civil nuclear power. It is natural that the accident at Chernobyl should have given the nuclear planners pause, but that is not the only reason why 28 GW of planned capacity has been stripped from the original plans during the past year (see page 298). Even before the appointment of Mr Mikhail Gorbachev as Party general-secretary, there had been protests in republics as different as those in the south and the north-west at plans to build nuclear power stations there, often apparently as if to canalize more general discontent in directions that could not be held to be illegitimate. The coming of *glasnost* has inevitably complicated the planners' lives, creating for Soviet agencies problems to which they are unused — those of winning advance public approval for projects that, in the old days, would have been decided on strictly administrative grounds.

Now they are in a real fix. Members of the Soviet public have been given both a foundation (the Chernobyl accident, and the hair-raising evidence of mismanagement that it revealed) and a mechanism (*glasnost*, which is not merely a licence for the press, but for local and regional Soviets as well) for dissent. The result is that opposition to the nuclear power programme is no longer simply confined to what are called intellectuals, or to republic nationalists (the Lithuanian government last year declined to contribute, as required, towards the cost of a fourth reactor being built at a site on its territory), but is much more broadly based. Moreover, local and regional Soviets, whose consent is formally required for developments in their spheres of influence, can now speak up and have an incentive to do so. (Self-interest will have their members calculating that unpopularity with constituents may lead to defeat in some future popular election, if the electoral system does indeed develop along democratic lines.) Worse still, the nuclear planners find that the Soviet press has emerged into *glasnost* with a distinctly anti-nuclear predilection, born partly of Chernobyl and partly of the appearance of complacency among the managers. The result is that an article by somebody in high authority in a sufficiently august newspaper will no longer suffice to turn public opinion around. Nuclear planners in the West would find themselves on familiar ground in the Soviet Union now.

What is to be done? The Soviet target of 100 GW of nuclear generating capacity by the year 2010 looks unattainable. There are optimists who believe that 80 GW might be managed, but even that target must depend crucially on what happens in the next few years. Yet on traditional planning assumptions, nuclear generating capacity on some such scale will be needed if intended industrial developments are to succeed. Moreover, most of this extra power will be needed in the western regions of the Soviet Union, which happen also to be where most people live, and are now able to dissent. The assumptions on which the planners' forecasts are based may, of course, be incorrect, but in the absence of market pricing for different kinds of fuel, there is no way of being sure. Similarly, in the absence of direct incentives for users (as distinct from planners) to economize in the use of energy, the Soviet Union cannot easily expect that fuel efficiency will take the edge off the growth of electricity demand, as it has done in the West since 1973.

So there is no choice but to win round public opinion — a daunting task requiring that the Soviet nuclear industry should be even more adventurous in its exploitation of *glasnost* than even the most adventurous of the Moscow newspapers. Experience elsewhere (Britain, for example) shows that people must be told of routine mishaps at nuclear plants (as in industrial enterprises of all kinds) in the hope that they may eventually

become familiar with the notion that the nuclear industry does not need to be literally free from accidents (but it has to be something of a paragon) to be safely manageable. It will be interesting, and may be instructive for the West, to see how brave the Soviet planners will be in these unfamiliar circumstances. But that they have little choice is beyond dispute. Meanwhile, in the search for nuclear generation sites, there seems little choice but to build new reactors at existing sites until the winds of dissent have abated. □

US budget pragmatism

The US budget problem is not its size, but how to cut it.

SENATOR Lloyd Benson's celebrated gibe at now-Vice-President Daniel Quayle, "You're no Jack Kennedy", might as well have been levelled at President George Bush last Friday, after his low-key inaugural address. But Bush is none the worse for that. Not every US president needs to sound like a Lincoln, a Roosevelt or a Kennedy, nor is rhetoric at the beginning of a four- or eight-year stint a guide to performance in the future. It may even help in present circumstances if the new presidency is seen to be manned by down-to-earthers who prefer to deal with real problems by solving them.

As yet, unfortunately, there are only straws in the wind as guides, and they seem to be blowing in contradictory directions. Everybody appreciates that US government support for research, like all its other discretionary expenditures, will be determined by the outcome of this year's tussle over the budget. As always, nobody will forget that this year's new congressmen (there are not many) will be campaigning for re-election before the next financial year (called fiscal 1990) is up. The best hope is that revenues can be modestly increased (preferably by natural growth, otherwise by devices that cannot this year be called taxes) and that expenditure can be selectively reduced. There are some signs of grace. The new National Security Adviser, the formidably phlegmatic General Brent Scowcroft, may have been less than enthusiastic about the Strategic Defense Initiative on television at the weekend: does that mean that a large part of \$5,600 million, three times the budget of the National Science Foundation, has been saved already?

On the other side of the coin, there is a general air about the new administration suggesting scepticism of President Ronald Reagan's commitments to the arms control processes begun in the past six years. It is true, of course, that the new president is much less sceptical than was Reagan on entering office, and that it is natural that a new administration should turn a quizzical eye on the deals its predecessors have been making with others, if only to be sure of understanding them. It would not be the end of the world if the Bush administration should conclude, for example, that it would prefer to abandon the proposed 50 per cent reduction of strategic arms in favour of some other accommodation with the Soviet Union and its allies in the Warsaw pact.

Yet those in Washington now musing aloud that Mr Gorbachev is interested in arms control and other accommodations only because of his domestic economic problems (and Gorbachev appears to admit that his budget is part of his motive) should reflect that the same calculation could apply to the United States. The most inflexible part of the US budget is that (nearly a third) spent by the Pentagon. The years immediately ahead will be especially difficult because development projects will be clamouring for procurement. Would it not be a prize worth having to be able to cut the defence budget without endangering security? The new administration is only a few days old, and there are many urgent matters clamouring for attention, but it will be a misfortune of this trade-off has not arisen naturally before the budget negotiations reach their climax later in the year. □

Sakharov defeated by "odd arithmetic"

- Absentees assumed to vote against
- Academy relinquishes five seats
- Sakharov nominated for Moscow City

Moscow

THE failure last week of the enlarged plenum of the Soviet Academy of Sciences to nominate Andrei Sakharov for the forthcoming election of Soviet deputies has caused great surprise and raises the question whether Soviet people, including men and women of science, yet know enough about democracy to practise it.

Sakharov, who had been supported by 60 of the academy's institutes, was not the only disappointed candidate. There were also Roald Sagdeev (supported by more than 25 institutes), Dmitri Likhachev (20 institutes) and such prominent economists (active advocates of *perestroika*) as Gavriil Popov (15 institutes plus), Nikolai Shmelev (more than 12), Gennadi Lisichkin and Abel Aganbegyan.

Last week's proceedings were an intermediate stage in the election of the 2,250 members of the Congress of People's Deputies, which will meet once a year to decide constitutional matters and will also elect the bi-cameral Supreme Soviet. A total of 75 seats in the chamber has been reserved for representatives of professional associations and societies, of which the academy had 25. But scientists can be elected by other categories of voters. Thus Guri Marchuk, president of the academy, and Yevgeni Velikhov, vice-president, have been nominated as candidates by the Communist Party.

The 17-member electoral commission took two hours to determine the outcome of the voting, by secret ballot. When the results were announced 10 hours after the meeting had begun, those left on the platform and in the hall were equally surprised. The outcome was so unexpected that it seemed like the dénouement of a whodunit.

One strange consequence is that there are now fewer contenders (23) than there were originally vacancies (25) in the academy's intended representation in the Congress of People's Deputies. It had been planned that there would be a meeting in two months at which the contenders would compete, promoting their own platforms. But the first outcome of the plenum would have made meaningless the continuation of the election of the academy's list: the contenders would have had no rivals.

Accordingly, the members of the plenum decided to cut the academy's representation first by two and then by five, a decision endorsed by the praesidium (governing council). The spare seats

will be filled by other academic societies and associations.

This development points both to the imperfections of the electoral machinery and to the likelihood that people do not fully appreciate their role in the democratic process.

This election is in two stages. In the first, nominations were made by the members of academy institutes and establishments. People at these meetings participated enthusiastically. Eventually there were more than 130 names on the list of those seeking the academy's nomination. Last week's plenum was intended to prune the list for a second stage at which choices would be made by all members of the academy and institute representatives (one for every 150 workers).

By prior agreement, the plenum did not discuss all the candidates because of lack of time. But it was agreed that the opinions of nominating institutes should be studied carefully, which is significant because the plenum thereby learned of the nominees' scientific and, more importantly, civic qualities. But the outcome of the plenum's procedural discussion was also to ensure a vote 'against' from each of its absent members.

Strictly, this accords with Soviet electoral law, which requires that those elected should receive "the vote of more than half the aggregate number" of those entitled to vote. Last week, 379 people were eligible to attend the enlarged plenum, but 102 of them were absent for various reasons. Whether the academy's election commission ever discussed with the central electoral commission the question of how the absent votes should be dealt with is unknown.

In the event, the qualifying quota was set at 139 votes, but only 190 people took part in the voting, so that the votes of the 89 people who did not were counted as 'against' in each case. Thus each contender had to secure 73 per cent of the votes to be elected. (Yablokov needed only one extra vote, but Sagdeev needed 18 and Sakharov 34.)

Much the same happened at the All-Union Society of Inventors and Innovators, where the same odd arithmetic meant that only six contenders instead of ten managed to secure qualifying votes. But that council reconvened the plenum to fill the 'vacancies'. Should not the academy have done the same?

One interpretation of the purpose of the academy's plenum is that it was to ensure

that the final choice of its 25 representatives would be genuinely competitive, reflecting the will of the scientific community at large. This might reasonably have been done in keeping with certain criteria, such as the number of institutes backing each candidate.

Many of those not nominated had solid backing. It goes without saying that Sakharov was the undisputed leader in the race he eventually lost. But Sakharov is extremely popular outside as well as within the scientific community, with the result that he was nominated, at a meeting on 20 January at the Lebedev Institute where he works, as a candidate for people's deputy from Moscow's Otyabrsky district and, two days later, as a candidate in the election of a deputy to represent the City of Moscow. **Yuri Kanin**

Novosti

Tajikistan struck by earthquake

London

THIS week's earthquake in the Soviet republic of Tajikistan, although not as serious as the Armenian catastrophe last month, has inevitably added to growing concern in Tajikistan about seismic safety.

The earthquake is the third in four years. There was a major earthquake in the north of the republic in October 1985 and the collapse of a dam at Sargazan in March 1987. Land and mudslides, such as that which this week buried two villages, are a common accompaniment.

What concerns the population most is the rapid development of hydroelectricity in the republic. A further eight dams are now planned on the River Vaksh, which already has the massive Nurek (305 m) and Rogun (345 m) dams. People fear that earthquakes may rupture the dams, and even that the dams themselves may trigger earthquakes.

At a meeting two years ago in the Tajikistan capital of Dushanbe, representatives of the Tajikistan Academy of Sciences revealed that the dams had been designed for seismic conditions appropriate to a plain, not a mountainous region, where the effects of seismic disturbances are usually greater. *Pravda* described the new Ziddin reservoir as "millions of cubic metres of water hanging like the sword of Damocles" over Dushanbe.

Tajiks themselves, who say they have little need of the electricity which is mostly exported to Afghanistan and other republics, seem in little doubt. A few days after the Spitak disaster, environmentalists and others formally petitioned for a halt to dam construction. **Vera Rich**

NIH gives the go-ahead on genetic experiment

The controversial first experiment to involve the insertion of foreign genes into humans — which has been deliberated by seven US review committees and government agencies over the past several months — received approval to proceed last week. James Wyngaarden, director of the US National Institutes of Health (NIH), authorized Steven A. Rosenberg and W. French Anderson to readminister interleukin-2-stimulated T-lymphocytes marked with a gene for neomycin resistance to ten cancer patients from whom the cells were isolated. The inserted gene will provide no therapeutic benefit to the patients, but will enable Rosenberg and Anderson to determine if the cells return to the tumour to fight the cancer. C.E.

Vet schools to close

THE University Grants Committee announced last week that two of Britain's six veterinary schools are to close. A working party appointed to consider the rationalization of veterinary education said that no school is at present able to teach clinical science with the necessary spread of disciplines and depth of coverage.

The schools at the Universities of Glasgow and Edinburgh will be merged to form a new school based in Edinburgh, and veterinary deg-rees will no longer be obtainable at the University of Cambridge. The last intake of students will be for the academic year 1989–90. The remaining schools, at the Universities of Bristol, Liverpool, London and Edinburgh, will have no fewer than 36 senior clinical teachers and will teach about 76 students each year. C. McG.

Scientists for Phobos

Ten US scientists, including Bruce Murray of the California Institute of Technology and Thomas Duxbury of the Jet Propulsion Laboratory, have been selected to participate in the Soviet Phobos mission, due to reach Mars and its satellite by April this year. The ten were chosen jointly by the National Aeronautics and Space Administration (NASA) and Soviet delegations to a US–Soviet working group — the working group was set up under the 1987 cooperative agreement on space research between the two countries.

Meanwhile a new agreement to foster scientific cooperation was signed in Paris earlier this month by outgoing US Secretary of State George Schultz and Soviet Foreign Minister Eduard Shevardnadze. In eight general areas, including science policy and problems of the Arctic as well as all the traditional disciplines, US agencies and their Soviet counterparts will draw up plans to facilitate cooperative research. D.L.

More criticism for NIH over 'summary' cut of grants

Boston

THE US National Institutes of Health (NIH) are drawing more fire for their decision to end financial support for two scientists accused of misconduct before an investigation into their case had been completed.

NIH procedures for investigating misconduct have recently come under heavy criticism. The current case involves two scientists at the Harvard-affiliated Massachusetts Eye and Ear Infirmary. Kenneth Kenyon and Scheffer Tseng (Tseng is now a researcher at the University of Miami) are alleged to have had business interests in an ophthalmic ointment they were testing with funds from NIH and to have delayed publication of results showing the ointment to be ineffective (see *Nature* 336, 506; 8 December 1988). NIH suspended their research grants late last year, but are still investigating the affair.

But Claes Dohlman, outgoing chair of the ophthalmology department, this week sent a letter to NIH officials expressing his concern over the agency's action and protesting at what he calls the "summary way" in which NIH reached their decision. Dohlman's letter follows a similar one earlier this month from Charles Schepens and Richard Pharo, respectively president and director of research administration at the Eye Research Institute in Boston.

"To take action in the absence of evidence is unjustifiable", write Schepens and Pharo, adding that NIH's move sends a message to the medical research community that "the federal government is prepared to abandon a scientist without due process". Last month, Representative John Dingell (Democrat, Michigan), chairman of the House of Representatives Energy and Commerce Committee oversight and investigations subcommittee, which has been holding hearings on how scientific misconduct charges are investigated, wrote to Otis Bowen, the Health

and Human Services Secretary, with similar complaints about the way the case was handled (see *Nature* 336, 706; 22 December 1988). Dingell, in a sharply worded letter, accused NIH of reaching their decision solely on the basis of newspaper reports and called upon NIH officials to explain the rationale and precedent for their decision.

In a letter responding to Dingell's concerns, Robert Windom, Assistant Secretary for Health of the Department of Health and Human Services, writes that NIH "acted responsibly" on the basis of the information they had at the time, which he states was "sufficient to demonstrate a clear, urgent need for interim administrative action". Windom claims that, in addition to newspaper accounts, the agency based its decision to terminate Kenyon's and Tseng's financial support upon conversations with Harvard officials and upon a statement written to the Harvard faculty about the case by Daniel C. Tosteson, dean of Harvard Medical School. Jack McLaughlin, associate director of the National Eye Institute of NIH, did acknowledge, however, that the agency first learned about the case through newspaper accounts.

Both Windom and McLaughlin assert that there are precedents for NIH's move, although neither points to any specific case. McLaughlin stresses the importance of treating such incidents on "a case-by-case basis", and says only that he is sure that similar "interim actions" have been taken in other cases at NIH.

A staff member on Dingell's subcommittee has called NIH's response "disingenuous" and "inconsistent" with the subcommittee's records of what transpired in NIH's decision. Hearings on the matter by Dingell's subcommittee are expected to begin in early March, and reports are due before that from at least one of NIH's investigations. Seth Shulman

Vice-chancellors' pay offer to staff rejected

London

UK university management last week tried temporarily to resolve the pay dispute with staff by offering to increase salaries by 3 per cent from April 1989 and make a lump sum payment at that time equal to a 3 per cent rise for the period from 1 January to 31 March 1989.

After making the offer, the Committee of Vice-Chancellors and Principals acknowledged that it was too low. Sir Mark Richmond, chairman of the committee, said it was embarrassed at the inadequacy of the offer. There was a desperate need to pay academic staff

decent salaries, but the committee thought staff would rather be assured of something now while it continued to argue for more. The vice-chancellors are to approach the government for extra money for 1989–90 which would be used by individual universities to reward merit and make discretionary payments to teachers in subjects short of staff.

The Association of University Teachers rejected the offer and will continue its boycott of university examinations. But it was cheered by the vice-chancellors' offer to backdate the offer to cover part of 1988–89. Christine McGourty

Guidelines produced for the use of transgenic animals in research

London

WITH transgenic animals finding increasing favour in both biomedical research and biotechnology, the UK Advisory Committee on Genetic Manipulation (ACGM) has produced detailed guidelines on their use. The guidelines, which the committee hopes will set an example for other countries, are particularly concerned with the consequences of the deliberate or accidental release of transgenic animals into the

wild, and the hazards of using viral, and especially retroviral, vectors for transgenes — that is, the use of genetically modified viruses to introduce new genes into animals.

ACGM, which will need advance notification of any plans to use viral vectors or to release transgenic animals, warns that it will be tough on both. For animals that are to be released, there will be a ban on the use of any viral vector that contains even a partially functional oncogene and on any form of vector that has even a remote potential to replicate. Plans to use equivalent vectors to produce contained transgenic animals will be considered individually.

Among the considerations will be the precautions that have been planned to ensure containment. For large animals, ACGM takes the view that a well-fenced field is sufficient. Extra care will be needed for fish and other aquatic vertebrates because of the relative ease with which they or their gametes can escape. Even greater precautions are necessary for work with invertebrates that "crawl, jump or fly".

Some of the guidelines on genetic manipulation anticipate changes in the official Health and Safety Regulations, which were drawn up in 1978. Revised regulations suggested by the Health and Safety Commission in October 1987 have since been modified, and now await approval by the Secretary of State and parliament. Their main intent is to establish a legally backed compulsion both to notify ACGM of the production and use of transgenic animals and to set up and operate local risk assessment committees at any establishment that carries out genetic manipulation.

ACGM's guidelines also cover aspects of work with transgenic animals that fall outside its own purview. The welfare of such animals is "dealt with adequately by existing legislation", the guidelines say, pointing out that breeding from transgenic animals is regulated as though an animal experiment until it can be demonstrated that the progeny are not likely to suffer any adverse effects.

The guidelines advise that the consumption of transgenic animals or their products by humans or animals would be subject to consideration by the Advisory Committee on Novel Foods and Processes, which may in turn seek advice from ACGM. Professor Mark Williamson, who chaired the working group that drew up the new guidelines, says it is inconceivable that approval would be given to the consumption of animals containing a human transgene.

Peter Newmark

Italian environmental programme launched

Turin

THE Italian government last week launched a £70 million programme of research into environmentally benign technology. Research priorities must be formulated within two months by a new commission which will report to the ministries for industry and for the environment which are financing the programme.

The announcement was well-timed, coinciding with an international conference in Turin designed to increase public awareness of the problems of the greenhouse effect, the destruction of the ozone layer and acid rain and to stimulate political action to tackle these problems. Among the recommendations of the conference was a proposal to impose a tax on fossil fuels which would finance a world fund to promote alternative energies and energy efficiency. The conference also recommended improved monitoring of climate change and increasing research into an understanding of the problems. The Italian government was heavily criticized for the lack of support for such research. Money is scarce and research uncoordinated, as well as being hindered by a lack of access to meteorological data, which is controlled by the defence ministry. Christine McGourty

HIV a problem for Indian blood banks

New Delhi

INDIA is suffering from a wave of public alarm caused by news that human immunodeficiency virus (HIV) is increasingly being transmitted by use of donated blood. This development conflicts with the earlier belief that AIDS is being spread chiefly by prostitutes and foreigners.

Officials say that the government can no longer postpone compulsory screening of all blood banks, a suggestion that only six months ago was dismissed as unnecessary as well as impracticable.

No transfusion-related HIV infection had been detected in India until July 1987, which is one reason the government was content with only minimal restrictions on imported blood products. But since then, the proportion of seropositive cases ascribed to transfusion has been increasing rapidly.

Last month alone, the All-India Institute of Medical Sciences in New Delhi discovered antibodies to HIV in three haemophiliac patients, and doctors in another hospital said that 10 of their 6,136 voluntary blood donors tested positive for AIDS. Of the 620 confirmed cases of HIV infection in India, about 60 have received blood transfusions in Indian hospitals.

According to the Indian Council of Medical Research (ICMR), which operates some 40 AIDS surveillance centres, blood transfusion has become an important mode of HIV transmission. An estimated 1.5 million units of blood are used each year in India, but ICMR's centres can perform only 30,000 tests, using kits imported from Britain. Complete blood screening would require a 100-fold increase in the number of kits. Although ICMR feels that all donated blood must be screened, the job "cannot be done overnight".

As a first step, all donors in Bombay and Madras are now being screened, and experience from these cities will be used to start screening in other cities in about a year, by which time India hopes to be producing its own test kit. K.S. Jayaraman

Testing of *in vitro* embryos approved

Melbourne

AN Australian parliamentary committee has approved a controversial request by scientists at the Monash Medical Centre in Melbourne to test human embryos obtained for *in vitro* fertilization (IVF) for birth defects before implanting them in patients.

Tests will be done about two days after fertilization, when the embryo has grown to four cells. One cell will be removed and analysed for genetic defects. The tests will be performed only on embryos that take longer than usual to fertilize. According to Monash Medical Centre's Professor Carl Wood, the Australian pioneer of IVF, about 3 per cent of embryos have a delayed fertilization, but are not necessarily abnormal. The tests will prevent normal embryos from being discarded. Embryo biopsy does not seem to damage the survival chances of the remaining embryo.

The decision of the committee, chaired by Professor Louis Waller of Monash University, may cause problems for Victoria Health Minister David White, who has written publicly that there will be no experimentation on human embryos older than 22 hours. The scheme may also face legal problems, as it involves the use of fertilized embryos but is not directly related to alleviating infertility.

Tania Ewing

UK research grants suffer from SERC's lack of money

London

FIGURES published this week in the Science and Engineering Research Council's corporate plan highlight the growing imbalance in supply and demand for UK research grants. Expenditure has increased slightly since the first plan was published in 1984-85, but costs have increased further and the council cannot support the increasing number of grant applications. The research grant success rate for alpha-rated proposals decreased from 72 per cent to 48 per cent in science and from 98 per cent to 86 per cent in engineering. For standard grants, the success rate decreased from 28 per cent to 20 per cent in science and 39 per cent to 32 per cent in engineering. The council hopes to increase the percentage of grant-in-aid spent on research grants from 33 per cent in 1987-88 to 40 per cent, and to maintain at a constant level the numbers of research assistants supported by its grants.

Expenditure on equipment must increase, and the council hopes to achieve this by increasing the proportion of grant money that supports equipment and by allocating for equipment money for the interdisciplinary research centres (IRCs). Five IRCs have been established and the council plans for another 17 by 1991. More than 300 applications to host the centres have been received. But the plans for expansion depend on the money available. Only 50 per cent of the cost can be met from existing funds, says the council. It will seek further money from the research

council's flexibility fund and from the Department of Education and Science.

The council plans to continue increasing the proportion of its budget devoted to strategic research with the aim of achieving a 60:40 ratio between support for strategic and for basic research by 1990-91. In the past decade, support for strategic research has increased from 42 per cent of total expenditure in 1978-79 to 54 per cent in 1987-88. For basic research, the council would like to make the support mechanism more long-term and to link expenditure to the country's gross domestic product.

Other council plans include an increase in expenditure on the LINK programmes, which are supported jointly by the research councils, government departments and industry. It plans to initiate eight more programmes annually over the next three years and to increase annual expenditure to £20 million in five years.

The council is also planning a collaborative programme of atmospheric research with the Natural Environment Research Council, to integrate satellite, ocean, Earth and atmosphere measurements with gas-reaction kinetic data and with related computer simulations.

Expenditure on space science will not be increased and, so that it might in future select parts of the science programme of the European Space agency in which to get involved, the council urges a review of ESA's method of operation to include some optional elements.

Christine McGourty

Most French pupils to go to university

Paris

A major overhaul of the French education system published last week by Education Minister Lionel Jospin aims to make France the best-educated nation in Europe by the year 2000. The plans, estimated to cost between \$8,000 million and \$160,000 million, include massive recruitment of teachers, better teacher training and a revised curriculum. The goal is that 80 per cent of school-leavers should be able to go to university, while the rest should not leave school without a diploma in business or industrial skills.

Education and research were priorities in President François Mitterrand's pre-electoral "letter to the nation" last spring. Despite a national curriculum, with 90 per cent of pupils attending state schools, only 40 per cent get the chance of a university education. Every year, 100,000 pupils leave school without any qualifications. Over one third of school leavers who do obtain their 'bac' (university entrance certificate) and go on to university fail to complete their course, many dropping out after the first year having failed the stiff examinations.

Jospin says that schools have an unwritten contract with children and their parents to provide the education they desire. One necessary step needed to fulfil this contract is to reduce class sizes. To this end, 300,000 new teachers are to be recruited over the next ten years. To make the profession more attractive, money will be provided to close the gap between the university-oriented 'lycées', and the less specialized, but also lower status, 'colleges', which are understaffed and have a high failure rate at the *baccalaureat*. Teacher-training institutes are to be set up and secondments introduced to allow teachers to improve their career prospects, as well as to catch up in their subjects.

The two main concerns with Jospin's plans are that success rates will be made artificially high by lowering pass marks, and that already overcrowded and crumbling universities could not cope with a large influx of students. Despite these objections, and some resistance from lycée teachers, the reforms have met with general approval.

Peter Coles

Soviet bloc research criticized

London

COMECON's Comprehensive Programme of Scientific and Technological Cooperation, the first major policy development of the Gorbachev era, has been roundly criticized on Prague radio. Comecon, the trade and development alliance of the Soviet bloc, is celebrating its fortieth anniversary this week.

The programme was intended, when launched in December 1985, to encourage joint enterprises between member countries and was regarded as a step towards decentralization. Previous policy had been heavily orientated towards the Soviet Union, with a Soviet "head" institute for every project. The best-known exception had been the low-temperature magnetism laboratory at Wrocław in Poland.

Radio Prague commentator Vladimir Vercak now says that within a few months of the adoption of the new programme, it

was clear that differences of economic management in member countries would prevent the goals of the programme being attained. Joint enterprises have been successfully established only where long-standing partnerships already existed, but the lack of an intra-bloc currency is also an impediment. Unless the economic conditions of member states are rapidly improved, he concludes, the programme will not realize "Comecon's scientific and technological potential".

A NATO conference last autumn showed that there may be other problems. Speakers with first-hand knowledge of bloc countries drew attention to the way that the lack of equipment and resources tempts scientists and engineers to seek collaboration outside the bloc, while enterprises are often more interested in preserving their chances in world markets than in the needs of its socialist partners.

Vera Rich

Correction

The high field test apparatus SULTAN at the Paul Scherrer Institute (see photograph in *Nature* 336, 338; 1988) consists of magnetic spools contributed by ENEA and ECN. The design of the facility and the cryogenic infrastructure was realized by the Paul Scherrer Institute. Euratom helped to finance both construction and running costs of the apparatus.

AAAS annual meeting draws largest crowd of decade

San Francisco

THE annual meeting of the American Association for the Advancement of Science (AAAS) in San Francisco drew nearly 10,000 scientists, journalists and members of the public, making it the largest, and according to its organizers, the most successful meeting of the decade.

But the annual meeting still lacks a clear identity, and efforts to move it closer to a mainstream scientific meeting were not an unqualified success.

Since a low of only 2,300 people attending the Los Angeles meeting in 1985, AAAS has worked hard to boost attendance at its meetings. One approach that outgoing AAAS president Walter Massey credits with attracting a broader audience to the meetings has been special scientific symposia. These symposia, requiring a separate registration fee, were first held three years ago. This year's symposia on plant molecular genetics and protein folding drew standing-room-only crowds in 500-seat ballrooms.

But in neighbouring rooms of equal size, scientific symposia covering similar basic science topics seemed empty by comparison. Twelve sessions over four days brought top scientists from around the country to San Francisco for sessions on cellular receptors, developmental biology and gene expression and retroviruses and oncogenes, but audiences were disappointingly small.

Corey Goodman, professor of biochemistry at the University of California at Berkeley, who co-organized the developmental biology session, says he was mortified to find only 60 or 70 people in a hall that holds 500 listening to a paper presented by Harvard's Philip Leder.

Goodman maintains that a similar talk would have drawn hundreds at any of the San Francisco area's research universities. Each of the scientific sessions had several scientific luminaries who would typically draw large crowds, but none was well attended.

Goodman also complains that after exhorting him to coax the best scientists to come to the meeting, often at their own expense, AAAS then did little or nothing to promote attendance at the sessions. Goodman says that others who were asked to organize special symposia were similarly disappointed by the results.

Massey says he is not sure why these sessions failed to attract larger audiences, and the problem will have to be sorted out before the next meeting.

The San Francisco meeting did have its own social issues. A polite demonstration by an AIDS activist group disrupted an awards ceremony for AIDS researcher

Anthony Fauci, director of the National Institute of Allergy and Infectious Diseases.

Outside the meeting, animal rights activists distributed leaflets and held posters protesting at the use of animals for scientific research. The AIDS protesters threw confetti as part of their protest against the red tape involved in approving new AIDS drugs, said Massey, a stark contrast to the 1971 meeting in Philadelphia at which protesters threw blood in an anti-war protest.

The most controversial session of the current meeting occurred on the last day, when J. Philippe Rushton of the University of Western Ontario presented a paper using anatomical, genetic and behavioural studies as a basis for claiming that blacks exhibit less sexual restraint, have lower intelligence and have more violent temperaments than Caucasians or Asians. When news of the Rushton talk began circulating among the 771 journalists registered for the meeting, Massey was brought to a hurriedly scheduled press conference to deny that AAAS supported Rushton's theories, but to emphasize that it supported his right to express his opinion.

As an organization, AAAS is in the midst of change. In addition to the annual meeting, AAAS's most visible role is as publisher of *Science*. But the organization also has active programmes in public understanding of science, arms control and human rights. From 1975 until 1987, William Carey served as AAAS executive director, and with an insider's background in the workings of Washington, Carey put AAAS in the thick of the US science policy debates.

Carey was replaced by Alvin Trivelpiece, who had previously been an assistant secretary at the Department of Energy where he was widely credited with convincing President Reagan that he should approve plans to build the Super-conducting Super Collider.

But Trivelpiece resigned late last year to become director of the Department of Energy's Oak Ridge National Laboratory. His leadership was too short to establish any clear direction for the organization. Former *Science* editor Philip Abelson is acting executive director while the search for a successor proceeds.

AAAS has embarked on a series of long-term reviews on where it has been and where it is going. Massey is confident that the annual meetings will regain much of the vitality they had in the 1960s and early 1970s, when AAAS became a focus for the intersection of science and society.

Joseph Palca

Nuclear plant presents unique problems

Boston

THE first attempt ever to decommission a commercial nuclear power plant reached a crucial milestone last month when the reactor vessel of the Shippingport Atomic Power Station was hoisted out of its underground site. The reactor's removal is perhaps the largest single step in the Energy Department's five-year, \$98-million project to decommission the power plant, located outside Pittsburgh, Pennsylvania, but many steps still remain.

Built in 1957 as part of the "Atoms for Peace" programme, Shippingport was the world's first nuclear plant to operate solely for the production of electricity. It closed in 1982, and work on dismantling it has been going on for three years.

The 72-MW reactor was constructed in a joint venture between the now-defunct Atomic Energy Commission (AEC) and a private utility, and the government agreed at the time of its construction to take charge of its decommissioning.

All other US commercial reactors will have to be decommissioned by the utility companies that built them, focusing industry interest on the Shippingport project. In the United States, 15 plants are expected to be ready for decommissioning by the year 2000.

The Department of Energy (DoE) wants to use the Shippingport reactor as a model programme for decommissioning. But some critics say that experience gained at Shippingport will have limited relevance to future decommissioning, and will only emphasize the obstacles others will face.

The most glaring obstacle is that in the United States there is still no high-level nuclear waste repository for spent fuel rods from commercial reactors, and virtually no place to deposit low-level waste. All three of the existing low-level facilities are close to capacity and nearing the end of their designed life-spans. For commercial reactors now and in the foreseeable future, disposal of radioactive refuse from a decommissioned plant would be impossible.

But even beyond the disposal problem, critics say that DoE is not making as much of the exercise as it could for testing and evaluating techniques needed in the future. They also object to DoE's decision to remove the reactor vessel for disposal in its entirety rather than cutting it into pieces on site as will be necessary for larger plants. Even with the reactor vessel's removal, the job is far from over. On the Shippingport site, two more years of work are expected.

Seth Shulman

Soviet nuclear power plant programme marks time

Moscow

THE sad accident at Chernobyl three years ago has cast a shadow on the nuclear power programme of the Soviet Union. Although the Minister of Nuclear Power Engineering, Mr Nikolai Lukonin, has only recently reaffirmed the government's commitment to nuclear generation as a means of meeting growing electricity needs, planned nuclear developments are now being resisted vigorously by the public and by local authorities in some republics.

The decision to close the two reactors of the Armenian nuclear plant near Yerevan, after a reassessment of the seismic hazards following last month's earthquake, is a further aggravation of the problems. One reactor will be closed on 25 February, the second on 18 March, after the expected peak of winter electricity consumption. Electricity supply in the Georgian republic will afterwards be tightly constrained.

Growth of nuclear generation capacity has been practically zero over the past year, with 45 reactors at 16 sites with a total generating capacity of 34.4 GW. Total generation last year, at 215 GWh, was 12.7 per cent of electricity consumption. But although new capacity is under construction at 15 sites, the building programme has been substantially pruned. A total capacity of 28 GW has thus been lost.

Nuclear construction projects have been stopped in Azerbaijan, Georgia, Armenia, Lithuania, Byelorussia, the Ukraine and in the south of the Russian Federation. And it can be confidently presumed that further nuclear power stations will be vetoed.

These developments create an awkward predicament for the Ministry of Nuclear Power Engineering, formed after the Chernobyl accident with responsibility for eradicating the consequences of the accident. The ministry is retraining personnel at all nuclear plants, revising construction and operating standards and improving safety arrangements. Lukonin now acknowledges a need for greater openness, and an open dialogue with the public. Trust is easily lost, but regained only with difficulty.

The Soviet government's decision to invite the international Operational Safety Review Team (OSART) to the Soviet Union for three weeks in December is part of the process of regaining trust. The team, including staff members of the International Atomic Energy Agency (IAEA) and independent experts from a dozen countries, made a detailed examination of the 1,000-MW pressurized water reactor at the Rovno power station in the

Ukraine.

The preliminary conclusions, given at a press conference in Moscow by Ferdinand Franzen and Morris Rosen, respectively head of the IAEA safety division and director of nuclear safety, are generally positive. The group acknowledged the competence of the operating staff, the priority given to safety (hardly surprising after Chernobyl) and that radiation shielding and ecological aspects of the plant's operation were up to international standards.

Among shortcomings at the Rovno plant, the group draws attention to the poor quality of some of the equipment arriving there (whose defects must be made good on site) and shortages of some modern instruments and automatic devices. The OSART team also plans to recommend in its final report that the station's senior executives should be given

more decision-making power.

Lukonin says that his ministry is taking steps to toughen requirements for site selection, the supervision of construction and the acceptance of equipment, while the ministry responsible for instruments says that it is making good the shortages which have been noted. The recommendation that station managers should have more authority, meant to secure greater flexibility over decisions, would presumably also encourage them to act more responsibly: Chernobyl showed that the over-meticulous tutelage of the higher authorities breeds complacency.

The immediate effect of the OSART mission on Soviet public opinion will be to demonstrate the government's readiness to follow the highest international standards in the operation of nuclear plants. There have already been similar inspections of 29 reactors in 16 countries, presumably with similar effects. The next inspection in the Soviet Union will be of the two 500-MW heat-supply reactors being built at Gorky.

Yuri Kanin
Novosti

More turmoil at German universities

Munich

DESPITE government approval in December of an emergency aid programme for the overcrowded West German universities (see *Nature* 336, 704; 22/29 December 1988), demonstrations and strikes continued after the Christmas vacation.

The situation in Berlin, where the president of the Free University had to call for police help to keep open laboratories in the medical faculty, seems to be the most serious. Students there who were not on strike had complained that they were being deprived of their right to study. Most medical students have since returned to classes, but students in other faculties remain on strike, demanding more say in governing the university and an improvement in the abysmal conditions caused by overcrowding.

There have been demonstrations and strikes at other universities in West Germany. In Munich, more than 30,000 students from both the Technical University and the Ludwig-Maximilian-University marched peacefully through the middle of the town demanding more low-cost student housing, more professors, more student grants and loans and the construction of a long-discussed subway line to the campus at Garching.

All parties in the Bundestag (parliament) called on 18 January for "relief" of the overcrowding, but without being specific. West German Education Minister Jürgen Möllemann (Free Democrat) said he would try to persuade the cabinet to add DM400 million to the DM2,100 million emergency programme voted in December.

Steven Dickman

Stock Exchange tries to stop research leaks

London

LEAKING to the financial markets of information in medical journals before their publication is causing concern in the London Stock Exchange. Results in the *British Medical Journal* (BMJ) of a report favourable to Glaxo's Zantac were leaked the day before publication, causing the company's share price to increase unexpectedly and more than 3 million shares to be traded that day. An investigation of the incident was initiated by the Stock Exchange's surveillance unit; no evidence of insider dealing was found.

Since then, the Stock Exchange has been considering how such incidents might be avoided. One proposal is that drug companies be asked to inform the Stock Exchange if information about to be published is price-sensitive; notice of this would then be released on the Stock Exchange's news service before publication.

At present, pre-publication copies are issued to journalists; this information is often leaked to analysts when journalists ask for comment on new results. The BMJ this month warned journalists receiving advance copies that it will not continue to supply copies to journalists found to have disclosed any information before publication. Analysts would prefer to receive information at the same time as journalists, but that will not put an end to leaking. It is the analysts' job, said one, to find out what is not in the public domain and to use that information.

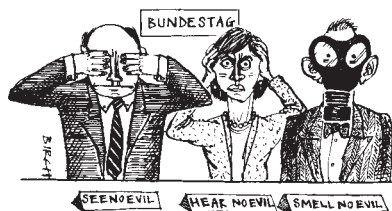
Christine McGourty

West Germany admits it knew of Libyan chemical plant

Paris

MORE evidence emerged last week that West German companies have been involved in the construction of an alleged chemical arms plant at Rabta, Libya, bringing a vigorous attack in the West German Bundestag (parliament) on 18 January of the federal government's handling of the affair.

Chancellor Helmut Kohl was accused of damaging West Germany's relationship with the United States, bringing shame upon the Bundestag, prolonging the threat of chemical arms and covering up the involvement of West German com-



panies in their manufacture.

For seven months, Kohl's government had shrugged off increasingly noisy US claims that Libya was building a chemical arms factory, and that West German companies were involved (see *Nature* 337, 199; 19 January 1989). But last week Chancellery chief Wolfgang Schäuble admitted to the Bundestag that both allegations were justified.

Schäuble confirmed that the government had been aware since May 1988 of the possible involvement of West German companies in building and supplying the

factory. But he defended the government's silence, saying that customs investigations of one company, Imhausen-Chemie GmbH, had failed to produce sufficient evidence for a prosecution.

Last July, West German customs officials were warned by foreign intelligence agencies that a Frankfurt-based company, Ihsan Barbouti International (IBI), was suspected of engineering an illegal supply route to divert construction materials and chemicals to Libya. But it is only this year that documents seized from IBI offices have confirmed the clandestine operations. The company is now in liquidation.

IBI, set up by Iraqi businessman Ihsan Barbouti, used a Hong Kong company that was also linked with Imhausen-Chemie to obtain legitimate export licences. But cargoes that included architectural plans from Imhausen-Chemie and ventilation equipment and dichloroethane from two other German suppliers were diverted to Libya.

Legal action is now being taken against IBI for falsification of export documents. But it is still uncertain whether the suppliers, including Imhausen-Chemie, Merck and Alfred Tewes GmbH, actually broke the law.

Despite tighter controls, the volume of export trade — about 2.5 million shipments a month from West Germany — makes it impossible to halt illegal trading completely. Now other governments are looking to see if they can point an untainted finger at West Germany.

Peter Coles

Japan denies involvement in Libya

Tokyo

JAPAN'S Foreign Ministry has firmly denied a US claim that two Japanese companies provided Libya with equipment for a chemical weapons plant. According to the spokesman, Yoshifumi Matsuda, the disputed "metallurgical complex" built in Libya by Japan Steel Works was designed to produce parts for a water purification plant, not shells for chemical weapons as had been claimed.

Allegations about Japan Steel Works and another company, Marubeni, hit the Japanese headlines last week after US Senator Jesse Helms included their names in a list of 36 companies, most of them West German, which he submitted to the Senate Foreign Relations Committee. All were alleged to have had links to the Libyan plant.

But Matsuda says that Senator Helms's list is based only on newspaper articles. In December, the *Christian Science Monitor* mentioned Japan Steel Works in an article

on chemical warfare. The Ministry of International Trade and Industry (MITI) has since had time to investigate the allegations and found them to be untrue.

According to a MITI spokesman, a close watch is kept on products that might be used to build chemical weapons, and the export of nine key chemicals from Japan is prohibited without special licence.

Alun Anderson

AIDS test law

BULGARIA has introduced tough new legislation aimed at halting the spread of AIDS. Unlike other socialist countries, where anti-AIDS legislation concentrates on curbing the sexual activity of people known to be HIV-positive, the Bulgarian law concentrates on compulsory testing and treatment. Heavy fines will be imposed on those who refuse tests, and also "persons who do not fulfil their official duties in the fight against AIDS".

V.R.

Another blow for creationism in California

Berkeley

THE California Board of Education has passed a policy statement on the teaching of science aimed at strengthening the message that evolution, not creationism, belongs in the science classroom. The statement supersedes a vague "antidogmatism policy" which often left teachers uncertain about how strongly they should stress evolution.

The antidogmatism statement, adopted in 1972 when Ronald Reagan was governor, was a brief directive on the discussion of "origins of life and earth", that required that "dogmatism be changed to conditional statements where speculation is offered as explanation for origins". California creationists, including members of the Institute for Creation Research in San Diego, have used the statement in their efforts to water down the teaching of evolution in California classrooms.

In 1981, a Superior Court judge decided against creationists in a case challenging the teaching of evolution as dogmatic. His decision affirmed that teaching of the scientific method is by definition not dogmatic, and that teaching of creation in public schools constitutes religious instruction, and is therefore constitutional.

But he upheld the antidogmatism policy as the ruler against which science education was to be measured. Scientists and teachers complained that the policy singled out the theory of evolution as somehow little more than conjecture, ignoring the true definition of a scientific theory and the fact that all science is based on theories that have stood the test of time. Many teachers, they said, felt compelled by the policy to couch their discussions of evolution in speculative terms.

The new policy statement carefully defines theories as tools of science, different from beliefs because they are logical constructs based on evidence, and subject to testing, modification and refutation. Beliefs, it implies, are closer to dogma, as they are based on ideology and not subject to test. A spokesman for the education board says that by requiring science teachers to teach only what falls within the definition of science, and directing administrators to support teachers in their resistance against pressure to do otherwise, the statement's drafters hope that it will provide firm support for science teachers who are put under pressure to teach creation in their classes.

The board is also planning to reject for use in California classrooms textbooks that do not deal adequately with evolution.

Marcia Barinaga

Lessons from eugenic history

SIR—Among the four papers on schizophrenia in the 10 November 1988 issue of *Nature*, there was one brief reference to an early work by the German psychiatric geneticist, Professor Ernst Rüdin^{1,2}. Unfortunately, there was no discussion of Rüdin's work or his career, subjects painfully relevant to the topic of the inheritance of schizophrenia. Rüdin was an internationally recognized psychiatrist-geneticist whose field of interest was the genetics of schizophrenia. In 1928, he became director of the Kaiser-Wilhelm Institute of Psychiatry in Munich. Rüdin was one of the principal architects and advocates of the Nazi programme of enforced eugenic sterilization, which was an application of his work on the inheritance of psychiatric disorders. The programme saw the sterilization of about 50,000 German citizens a year, beginning in 1934. It established a 'scientific' basis for the mass control of reproduction with the objective of eliminating undesirable traits of human life that were considered to be hereditary³. Criteria for sterilization in Rüdin's institute included being a conscientious objector, a frame of mind that was considered to be a form of schizophrenia and consequently classified as hereditary⁴.

Rüdin's genetic/eugenic fervour was not confined to compulsory sterilization. He was a supporter of the 1935 Nuremberg race laws, which he considered to have been an achievement for his eugenics movement⁵. He served as a member of a group of psychiatric 'expert witnesses' for the euthanasia programme of medicalized murder which, the 'experts' felt, would "meet with general understanding and approval, as it becomes established and more generally known that. . . all possible measures were taken either to cure the patients or to improve their state sufficiently to enable them to return to work which is economically worthwhile. . ."⁶.

To the uninformed, psychiatry appears to be a naive 'Cinderella' specialty that is only now joining the ranks of experimental genetics. The reality is that psychiatry has been a wicked 'step-sister' in the field of experimental genetics, a nefarious role which Ernst Rüdin helped to define. What is new today is the sophistication of genetic research which enables science to explore the molecular basis of human heredity in a manner undreamed of in the days of Rüdin. What is still needed, however, is a process that ensures that human values are appropriately applied with a sensitivity and a sophistication that takes into consideration the recent history of science and medicine where values and science were distorted to the detriment of all mankind. By ignoring this history, science has demonstrated that it has yet to

achieve the sensitivity required to pursue this type of research in a manner which is in the best interests of human society.

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Suffer the animals

SIR—If G.E. Lamming's account of an international symposium on biotechnology in growth regulation (*Nature* **336**, 19–20; 1988) is comprehensive, as I have no doubt it is, it implies that the subject of animal welfare was not considered seriously at that symposium. The absence of a session on the welfare aspects of genetic engineering reinforces the impression that little more than lip service is being given to the humane aspects of what is a headlong rush for quick profits by the meat-production industry.

It is clear from observations of transgenic animals so far produced that they frequently develop unexpected abnormalities that cause them considerable suffering. Indeed, Lamming mentions that transgenic pigs made by researchers at the US Department of Agriculture suffered high mortality and morbidity.

Only an elementary knowledge of molecular genetics is necessary to predict that the random insertion of foreign DNA into an animal's genome is likely to cause abnormalities in at least some of the recipients that will lead to real suffering. It is important that those involved in the production of transgenics should be seen to be taking particular care over this aspect of their work, for example by including it on the agenda of international meetings.

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Farewell Soil Bureau

SIR—As a result of an institutional rearrangement by the Department of Scientific and Industrial Research (DSIR), the New Zealand Soil Bureau has disappeared. After more than 50 years of carrying out soil research and providing soil maps, it has ceased to exist as an independent unit. Founded when the New Zealand economy depended almost

entirely on the soil, it has succumbed to the new style of New Zealand life and the reforming zeal of the government.

Probably its greatest triumph was the detection of the lack of critical trace elements in volcanic soils, which allowed successful sheep farming over wide regions of the North Island; and more recently the painstaking investigation of aspects of the Abbotsford landslide gained the admiration of the geotechnical community.

Soil science has now been incorporated into the new DSIR Division of Land and Soil Science, but the rationalization has meant that more than 20 scientists have lost their bureau jobs, including Benny Theng, Graham Claridge, Pauline McColl and Iain Campbell. Soil science in New Zealand has been diminished, as it has in the United Kingdom. It seems odd that as we become aware of major 'green' issues, agencies directly involved with their investigation are closed.

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Credit where it's due

SIR—In their 'anniversarial' article (*Nature* **337**, 29; 1988) W. F. Bynum and J. L. Heilbron, discussing the cell theory of the German botanist Theodor Schwann (1810–82), say that the famous phrase *Omnis cellula e cellula* "came later". Thus, by inference, they sustain the widely held but erroneous attribution to Rudolf Virchow (1821–1902).

In fact, and in print, it was first used by François-Vincent Raspail (1794–1878), as epigraph for his thesis "Développement de la fécule dans les organes de la fructification des cereales" (1825)¹, in which he refers specifically to the origin of new cells by division. The anatomist Matthias Schleiden (1804–81), Schwann, and later Virchow all appear to have lifted one or more of Raspail's prior contributions to the cell theory without acknowledging their source. The nationalistic nature of their behaviour is epitomized by a ringing declaration made in 1866 by the surgeon Pierre-Paul Broca (1824–80), that "the cell is French and belongs to M. Raspail", (quoted by Wiener, 1968)². But Broca was correct. It is time to remedy a gross injustice and to give Raspail, who is incidentally the undisputed founder of the science of histochemistry, his due recognition as father of the cell theory.

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European VLBI in limbo?

The failure of an important and worthy application for research funds to the European Commission demonstrates that Europe needs a more imaginative way of backing research that matters.

SOMETIMES, if rarely, there are research proposals that make perfect sense: the project is sound, indeed imaginative; the applicants have splendid track records, and are prepared to commit their own resources to the project; and there is every reason why the intended source of funds should welcome the proposal both as a vehicle for spending money wisely and because it furthers that organization's wider objectives. What executive officer of a grant-making organization could fail to jump at such a prize? Yet that is what the European Commission has just done.

The circumstances, which are not particularly complicated, concern the development of Very-Long-Baseline Interferometry (VLBI) in Europe. Everybody knows how valuable this technique has been shown to be in the past decade and a half. The principle is simply that of simultaneously pointing two or more radiotelescopes at the same distant radio source. The coordinated instruments then function as one instrument whose resolving power is proportional to the distances by which the instruments are separated (or, strictly, to the ratio of the baseline and the wavelength at which observations are made).

The principle is no different from that in which neighbouring radiotelescopes are deployed in concert to form a radiointerferometer, but there is a practical difference: because of the distances between the instruments, signals from different receivers cannot be compared in real time, but must instead be recorded on magnetic tape and correlated with each other later, using a device known as a correlator.

By now, the achievements of VLBI are widely appreciated. In the observation of distant radio galaxies, the technique promises the most direct observational evidence of what happens near the galaxy-sized nuclei of these often vastly extended objects, thus helping to decide whether the central galaxies are driven by black holes or by some other mechanism. Last year, an observation of the active nucleus of the radio galaxy 3C84 reported an accuracy of 100 microarcseconds (Bartel, N. *et al.* *Nature* **334**, 131; 1988), no doubt still a record. But there are many more familiar astrophysical processes, such as the mapping of star-forming clouds of gas and dust within our galaxy and the study of the movement of matter near peculiar binary stars, which have been made intelligible by the use of VLBI.

All this was expected at the outset. The surprise has been the usefulness of VLBI in geodesy and geophysics. Coordinated radiotelescopes can so accurately measure the orientation of distant radio sources relative to the Earth's surface that VLBI measurements have become standard measures of the motion of the Earth's poles (including the Chandler Wobble) and of variations in the length of the day.

The point has also been reached at which VLBI is yielding, in what might be called real time, a log of the relative movement of the Earth's tectonic plates. The uncertainty in baseline measurements, inferred from the observation of known distant objects, is already no more than 3 cm, comparable with the annual separation of the plates on either side of the Mid-Atlantic Ridge, and should be further reduced as more high-frequency instruments join the VLBI collaborations and as data analysis is improved. Already pairs of movable radiotelescopes have provided a plausible account of the internal distortion of the tectonic plate of the south-western United States.

But these developments are only a beginning. Coordinated observations by telescopes separated by the diameter of the Earth are commonplace, but now there are ambitions — even plans — to surmount the limitation of the Earth's finite size. The European and US space agencies have made a joint study of an orbiting radiotelescope, while there is a possibility that the Soviet Union may launch such a device in the early 1990s, as may Japan. VLBI, it seems, is booming.

That, sadly, is where recent European disappointments are ironical. From the outset, European radio-observatories have played an important part in wider international collaborations. Most of them now belong to a club, called the European VLBI consortium, which organises collaborations and worries about data acquisition and analysis.

There is the rub. For at least the past three years, it has been clear that existing facilities would soon be overwhelmed by data. Technically, there are two problems — that of recording data accurately in the expected volume (determined by the need to record with high time-resolution and over a range of frequencies) and that of comparing data from different stations so as to extract meaningful information.

This is what prompted the consortium last year to apply to the European Com-

mission for funds with which to equip itself with suitable facilities. The hope had been that a correlator centre could be founded at the Westerbork observatory in the Netherlands, and that the Commission might also help equip individual stations with suitable recording equipment.

As might be expected, the Commission seems to have warmly applauded the proposal, as neat a marriage as there could be of scientific merit with the ideal of European collaboration. Much in the spirit in which censors about to ban books first read them through a second time, the Commission cannot be faulted for the attention which it has given the proposal. But the plan falls outside the terms of reference of the most relevant of the Community's research programmes, the Framework stimulation programme, which, administered by the CODEST committee, exists to bring European researchers together in research collaborations.

In grant-makers' terms, there are several pitfalls buried in the VLBI correlator project, not the least of which is that CODEST's own funds are repeatedly being overtaken by growing demands. But the VLBI correlator would also be a permanent institution, with annual running costs, of a kind that the Commission is not at present equipped (or even empowered) to shoulder. So everybody has been sympathetic, and CODEST has offered the consortium a grant of 300,000 ECU (which it should accept) to improve on its proposal, or even to commission the design of a data-recorder.

This is a sad state of affairs for which nobody is to blame — as yet. While member states delight in playing cat-and-mouse with the Commission over its science budget, it is natural that the Commission should shy from building institutions with implicit promises to support them. It is similarly natural that members of the consortium should obtain from national sources the equipment needed to remain in membership. And nobody can complain that the Commission should seek to make its funds go as far as possible by concentrating on "pump-priming seed-corn projects", to mix two grant-makers' metaphors.

But when, as on this occasion, it finds itself having to turn down a project of outstanding merit simply because its own rules are inappropriately designed, it should do the next most sensible thing — and change the rules.

John Maddox

NMR spectroscopy

Beyond the magic angle

J.M. Thomas

A RECENT technical achievement¹, which entails rapidly spinning a sample in a magnetic field around two axes at the same time, like a top within a top, promises to revolutionize the structural elucidation of solids by nuclear magnetic resonance. Double-rotation NMR is the term coined by the originators of this significant development, A. Pines (Lawrence-Berkeley Laboratory), E. Lippmaa and A. Samoson (Institute of Chemical Physics and Biophysics, Tallinn, Estonia). It should greatly extend the range of magnetic-resonance spectroscopy because it improves, by about two orders of magnitude, the accuracy with which the siting of certain types of atoms and ions in a solid may be distinguished.

NMR had humble beginnings, at least so far as those interested in the determination of molecular structure were concerned. Shortly after its invention in the mid-1940s, it seemed that NMR would be of premier concern to those whose ambitions were accurately to measure the fundamental properties of atomic nuclei. Then came the realization, greeted with growing excitement by chemists, that the precise frequency with which a given nucleus (of finite spin) resonates in an applied magnetic field is a delicate function of the local electronic environment of that nucleus. This gave birth to the notion of chemical shift. For example, protons (which are spherical, spin 1/2), resonate at frequencies governed by the chemical groupings to which they are attached: the resonating frequencies of $-\text{HCH}-$, $-\text{CH}=\text{CH}_2$, $-\text{C}\equiv\text{CH}$ and $-\text{CO}-\text{OH}$ are very different from one another. For other spherical nuclei the range of chemical shifts is large; and ^{13}C NMR readily distin-

guishes between aliphatic and aromatic carbons, just as ^{29}Si spectra discriminate between the 4- and 6-coordinated element. This is one of the main reasons why NMR is an indispensable structure-determining tool in the physical and biological sciences.

In NMR spectroscopy one measures, *inter alia*, the radio frequencies emitted by, and the rates of realignment of, nuclear spins in a magnetic field after they absorb energy from radio-wave pulses. Because of random motion executed by entities in the fluid state, anisotropic magnetic and electrical interactions are averaged out so that high-resolution spectra may be routinely obtained with liquids and solutions. With solid samples there is no such averaging and the NMR spectra are hugely broadened. For a long time the prospects of retrieving chemical shift information from solid-state NMR spectra looked bleak until, in 1958, Andrew *et al.*², at the University College of North Wales, and Lowe³ realized that the blurring effects of spectral resonances are represented mathematically by a term that contains the factor $(1-3\cos^2\theta)$. By spinning the sample about an axis at an angle $\theta = 54.74^\circ$ (equals $\cos^{-1} 1/\sqrt{3}$) to the magnetic field, the solid behaved as if it were an isotropic liquid, and high-resolution spectra of a quality comparable to those achievable with liquids could be obtained.

Although magic-angle-spinning (MAS) NMR has been of enormous value in recent years in the study of solids^{4,5} in general, and of absorbents and catalysts⁶ in particular, as well as of a wide range of quasicrystalline and membranous materials — for which competing techniques such as X-ray crystallography are not as well suited — it has hitherto suffered from one rather serious limitation: quadrupolar nuclei (non-spherical ones, with spin greater than 1/2, like ^{11}B , ^{17}O , ^{23}Na and ^{27}Al), which are more numerous than spin-1/2 nuclei, give rise to broadening influences that are not eliminated by spinning at the magic angle of 54.74° nor by resorting to other strategies such as radio frequency manipulation of the spins.

This has meant that many problems of great interest to the solid-state chemist and physicist and to the materials and earth scientist have remained beyond the reach of high-resolution solid-state NMR. One practical, though costly, way of minimizing this limitation is to employ ever-higher magnetic fields, as second-order quadrupolar broadening effects are inversely proportional to the field strength.

This approach has indeed been successful⁷ in the study of zeolites, for which ^{27}Al MAS NMR spectra recorded at 11.744 tesla, but not at lower (7.946 or 9.359 tesla) fields, resolve two crystallographically distinct tetrahedral framework sites.

But double-rotation NMR offers an alternative route forward. By employing two 'magic angles' — the customary one of 54.74° and a new one of 30.56° , appropriate for the quadrupolar nuclei — essentially all the blurring effects that broaden the resonances are eliminated as a result of the complex motion to which the sample is

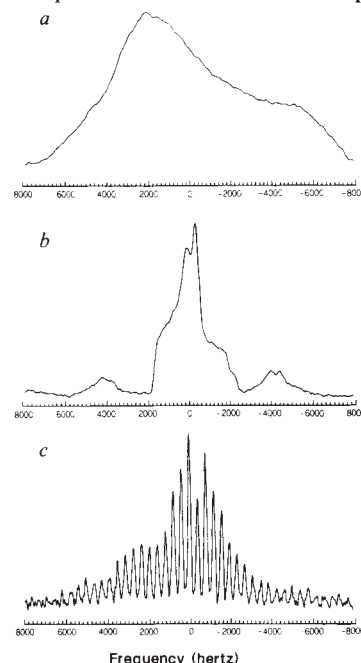


Fig. 2 The NMR spectrum of ^{23}Na in polycrystalline sodium oxalate, from a static sample (a), a magic-angle-spinning experiment (b) and a double-rotation experiment (c). The linewidth is reduced from 12,000 to 4,000 to 140 hertz, respectively, as dipolar and quadrupolar broadening are suppressed. The sidebands in b and apparent 'structure' in c are related to the spinning frequencies. (From ref. 1.)

subjected (Figs 1 and 2). The powdered solid rotates inside a holder which simultaneously rotates on a second axis, air jets and air bearings being used to achieve rotation speeds of up to 6 kilohertz.

The Berkeley-Tallinn team has already demonstrated that the technique works for ^{23}Na nuclei, two well-resolved signals (of width 75 hertz) being obtained from a mixture of the sulphate and oxalate of sodium. With the very recent report (A. Pines *et al.*, preprint) that the mineral diopside (isotopically enriched) yields three distinct ^{17}O signals, the stage is now set for exciting developments in the elucidation of structures and mechanisms of interest to the earth scientists, materials engineers and those investigating catalysts, adsorbents and warm superconductors. There is a paucity of techniques capable of probing directly the precise electronic nature of oxygen ions in such solids.

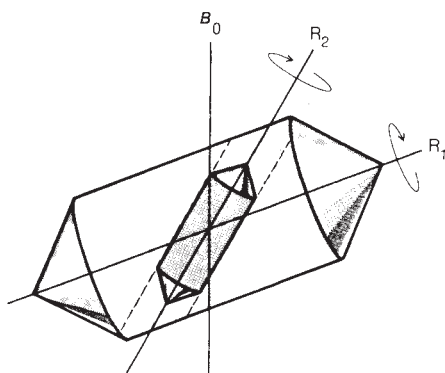


Fig. 1 In double-rotation NMR, the solid sample is rotated at an angle to the axis of the holder which is itself spun at an angle, the magic angle (54.74°), to the applied field B_0 . The angle between R_1 and R_2 is 30.56° . Together these rotations remove dipolar and quadrupolar line broadening. (From ref. 1.)

Electron-energy-loss spectroscopy (oxygen K-edge structure), X-ray spectroscopy and electron spin resonance are rather specialized though quite useful tools. But ^{17}O double-rotation NMR is potentially more powerful.

For zeolite- ρ , faujasite (zeolites X and Y) and ferrierite, all of which are important solid catalysts, there are known to be, respectively, two, four and eight crystallographically distinct oxygen sites. Will these be resolved by double-rotation NMR (like the 24 silicon sites⁸ in the ^{29}Si MAS NMR spectrum of ZSM-5)? And will there be only one peak in the ^{17}O double-rotation NMR spectrum of sodalite? Or more, if there are Al-O-Al linkages⁹ present? These answers will determine the new directions which solid-state NMR will give to the structural elucidation of new zeolitic and related catalysts. Likewise, with ^{17}O enriched superconducting

ceramics, apart from identifying crystallographically distinct oxygens, one may hope to find direct evidence for the so-called charge-disproportionation mechanism ($\text{O}^{2-} + \text{O}^0 \rightarrow 2\text{O}^-$) or the existence of the previously postulated peroxy, ionic species, O_2^{2-} . These issues need to be clarified if we are to understand the transport mechanisms in the various families of oxide superconductors. □

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Telomerase

The beginning of the ends

Jack W. Szostak

EVERY time a linear chromosome is replicated it is in danger of being whittled away from both ends. The origin of this danger lies in the requirement of DNA polymerases not only for a template to copy but also a primer to extend: because polynucleotide synthesis can proceed in only one direction, from 5' to 3', this leads to a problem in copying the 3' ends of both strands of a linear chromosome, where there can be no primer to extend (see figure). A common solution to this problem is to have simple repeat sequences at the telomeres (ends) of linear chromosomes, and a specialized system for generating additional repeats, compensating for loss in replication. Greider and Blackburn isolated and characterized^{1,2} an enzyme that can synthesize telomeric repeats; notably, this telomere terminal transferase has an essential RNA component. The same authors, on page 331 of this issue³, find that the RNA component of the telomere terminal transferase of *Tetrahymena* contains the complementary sequence of the telomeric repeats that it synthesizes, strongly suggesting that the enzyme is a remarkable kind of DNA polymerase that carries its own template.

The existence of telomerase was originally hypothesized⁴ to explain several bizarre properties of telomeres. Synthesis of new telomeric repeats has been observed in three very different organisms. In *Tetrahymena* and other ciliates, chromosomes are fragmented and new telomeres are formed spontaneously during the formation of macronuclei. In yeast, linear plasmids made *in vitro* with *Tetrahymena* telomeres can be elongated *in vivo* with

newly synthesized yeast telomeres. And in trypanosomes, telomeres elongate steadily at about one repeat per generation during somatic growth. Of course, telomerase also provides a ready explanation for the maintenance of normal telomere length in the face of incomplete replication.

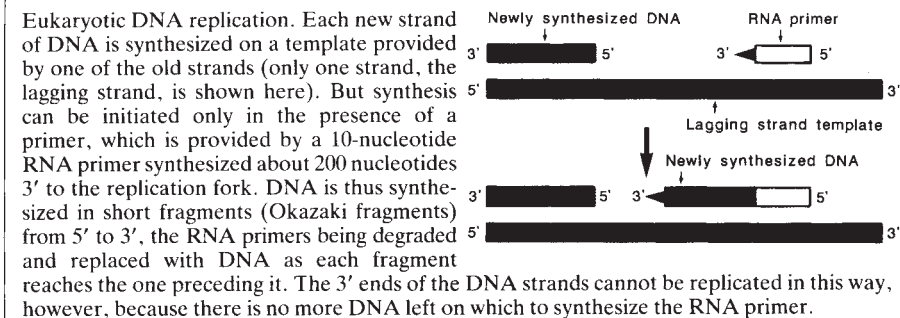
The *Tetrahymena* telomerase is a ribonucleoprotein that adds GGGTT repeats to short, single-stranded, synthetic oligonucleotides. Only telomere-like sequences are elongated; random-sequence oligonucleotides are ignored by the enzyme. How is this specificity obtained? Greider and Blackburn¹⁻³ cloned and sequenced the RNA component of telomerase, and find embedded within it the sequence CAACCCCAA — one and a half repeats of the complement to the synthesized sequence. Furthermore, they show that the enzyme can be inhibited by oligonucleotides complementary to this sequence, and inactivated by RNaseH cleavage at this site. Even more remarkably, an oligonucleotide that lines up just 5' to this sequence, but contains no telomeric repeats itself, can be elongated by

the enzyme, further implicating the RNA as a template³. No doubt we shall soon see the sequences of corresponding RNAs from related organisms with different telomeric repeats; if the RNA does indeed act as a template, each one should contain the appropriate complementary sequences. An even more decisive experiment would be possible if such an RNA template could be found in yeast: replacement of the resident gene with one modified to change the template sequence should generate yeast with any desired telomere sequence.

But do all eukaryotes replicate their telomeres with the aid of telomere terminal transferase? Similar enzymes have been detected in other ciliates — *Oxytricha*⁵ and *Euplotes* (Shipper-Lentz, D. & Blackburn, E.H., submitted) — so the role of this enzyme in these organisms seems assured. But it has not yet been detected in yeast, despite several attempts to find it. This is not necessarily surprising — the ratio of telomeres to total genomic DNA is about 1,000-fold less in yeast than in ciliates, so there may simply be very little of the enzyme in yeast. Still, most eukaryotes never need to generate telomeres *de novo*, and might perhaps be able to use some sort of recombinational process to elongate them. In such models, a telomeric 3' end could invade at an internal site in another telomere, where it could be elongated by polymerase on the existing template. Such a gene conversion-like process could lead to the same elongation as is achieved by telomere terminal transferase. Indeed, recombination is almost certainly responsible for maintaining the average telomere length of *Tetrahymena* mitochondrial DNA⁶.

Does recombination actually play a role in telomere elongation in yeast? Pluta and Zakian⁷ have done a cleverly designed experiment in which they prepared hybrid linear DNA molecules with CCCC AAAA repeats at one end and CCCC AA repeats at the other. After transformation into yeast, each end is elongated with yeast C₁₋₃A sequences, as expected. Hybridization analysis of the resulting linear plasmids reveals that in many cases CCCC AAAA sequences have been transferred to the CCCC AA end, and vice versa.

How has this happened? Pluta and Zakian suggest that recombination between the ends has taken place and,



furthermore, that similar recombination events involving yeast telomeres could be responsible for the subsequent addition of the $C_{1-3}A$ repeats, and for telomere elongation in general. Because their observed events are independent of the *rad 52* gene product, which is required for all other known recombination events involving double-stranded ends, some new, perhaps telomere-specific, system could be involved, if indeed recombination is responsible for these events.

Work from my laboratory argues against an essential role for recombination in telomere elongation⁸. We were surprised to find that 20–60 base pairs of non-telomere DNA can commonly be retained in between CCCAA repeats present on the input DNA and the $C_{1-3}A$ DNA added on in yeast. Because these linker sequences are unrelated to telomeric repeats, the subsequent addition of $C_{1-3}A$ repeats cannot involve homologous recombination.

Our results also provide an alternative explanation for the results of Pluta and Zakian⁷. A resolution reaction that can separate ligated telomeres is well-documented in yeast⁹. If the two different telomeres became ligated in yeast, and

then resolved by breakage within one of the stretches of telomeric repeats, the observed movement of DNA would take place. Pluta and Zakian reject this explanation because the transferred segment would be inverted relative to the normal telomeric orientation. Telomeric sequences in the wrong orientation are known not to be elongated in yeast. In the light of our results⁸, however, it is likely that a short stretch of inverted telomere DNA would not block subsequent elongation. The only way to resolve this point is to clone and sequence several examples of the chimaeric telomeres to determine the orientation of the transferred segment. □

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Molecular evolution

What, if anything, is *Prochloron*?

David Penny

It is almost 20 years since the suggestion¹ that free-living photosynthetic prokaryotic organisms containing chlorophylls *a* and *b*, but lacking phycobilin pigments, existed a billion years ago. These hypothetical organisms would be ancestors of the chloroplasts of green plants. At that time, the notion seemed reasonable, but scarcely testable. I still remember the excitement five years later when a possible member of such a group was found alive and well, living in Baja California, Mexico². The organism was named *Prochloron*, and the group prochlorophytes. But the first work³ comparing fragments of nucleotide sequences from ribosomal RNA of these organisms did not support prochlorophytes being the ancestor of green chloroplasts. Rather, it seemed that chlorophyll *b* had developed separately in *Prochloron* and chloroplasts. Progress in clarifying the position of *Prochloron* has been slow because it cannot be grown in culture. Recently, a free-living form *Prochlorothrix*, a presumed relative of *Prochloron*, has been discovered and cultured⁴. On pages 380 and 382 of this issue^{5,6}, two groups report on the evolutionary position of *Prochlorothrix* but reach apparently conflicting conclusions.

Turner *et al.*⁵ compare the nucleotide sequences of the small-subunit (16S) RNA from *Prochlorothrix*, chloroplasts

and cyanobacteria. They use distance methods for building evolutionary trees and estimate the accuracy of their results by 'bootstrapping'. Their trees clearly separate green chloroplasts from *Prochlorothrix*, in agreement with the earlier results³. In this scheme, the closest relative of a chloroplast ancestor would be the 'chloroplast' (cyanelle) of the protist *Cyanophora*, which has typical cyanobacterial structure and biochemistry. The interpretation is that the loss of phycobilins, and the gain of chlorophyll *b*

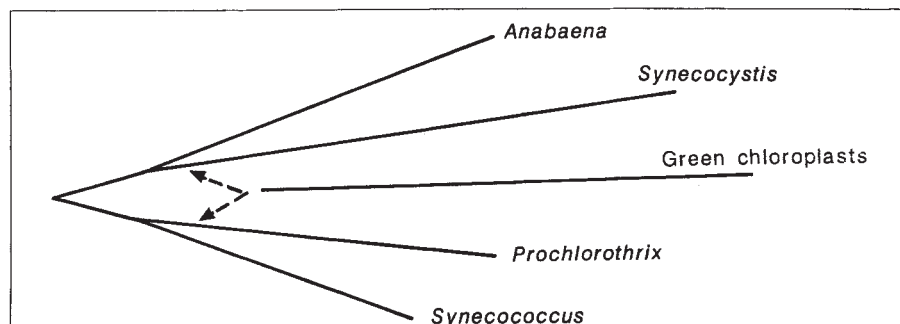
has occurred at least twice.

Morden and Golden⁶, on the other hand, use DNA sequences from a photosynthetic membrane protein (*psbA*) from photosystem II. They use the position of deletions in the molecule as well as trees built using the parsimony (minimum mutations) criterion and come to the opposite conclusion, namely that the prochlorophytes such as *Prochlorothrix* are the ancestors of the green chloroplasts. Similarities and differences between the reports^{5,6} are shown in the figure.

Such an impasse demands comment. First, neither group supports the idea⁷ that prochlorophytes are a very ancient group that evolved at the same time as cyanobacteria. It is small consolation that at least the two sequences put *Prochlorothrix* into the same main taxonomic group. Putting aside for the present special pleading such as lateral transfer, can we predict which conclusion will eventually be proved correct? Unfortunately, no.

Two strengths of the method of Turner *et al.*⁵ are bootstrapping to give an estimate of errors and a correction for unequal rates of change on different nucleotides. But the method could be sensitive to unequal rates on different lineages⁸. Turner *et al.* have two sets of biochemical data, RNA sequences and photosynthetic pigments (including information on biosynthetic pathways and chlorophyll-binding proteins). If the two sets of data do agree then it is valid to say they support the same tree. If they do not, then it is difficult to justify ignoring completely one or the other. The authors appear to assume their RNA tree is correct and give a zero weighting to the pigment data.

Another possible difficulty is that there could be some convergence of function in 16S RNA sequences in chloroplasts. This could lead to the *Cyanophora* and chloroplast sequences converging sufficiently to come together on the tree. An example of functional convergence has been found with lysozyme in two groups of mammals



The reports of Turner *et al.*⁵ and Morden and Golden⁶ have five 'groups' in common, three standard cyanobacteria, *Prochlorothrix* and chloroplasts from green plants. Assuming the differences in isolates are unimportant, the first four give the same rooted tree for 16S RNA and the *psbA* gene. Thus the difference is not in the position of *Prochlorothrix* but rather in the green chloroplasts. The 16S-RNA results⁵ derive the chloroplasts from near *Synechococcus*, which requires chlorophyll *b* to have arisen twice as a photosynthetic pigment. The *psbA* results⁶ derive chloroplasts from 'prochlorophytes', thus giving a single origin of chlorophyll *b*.

with ruminant digestion, ungulates (including cattle) and Langur monkeys⁹. If convergence has occurred, then one test is to run the RNA data without *Cyanophora*. *Prochlorothrix* should now join with chloroplasts. But there is other evidence¹⁰, observations of the ultrastructure, for example, that could relate the *Cyanophora* group to the green plants and support the tree of Turner *et al.*. It would also be interesting to determine the sequence of some anomalous protists that have chlorophyll *b*, such as *Chlorarachnion*¹¹.

Morden and Golden⁶ find that the two types of biochemical data do agree, but apart from omitting nucleotides in the region of the deletion, they do not test the accuracy of their tree. Parsimony methods are susceptible to error from long edges coming together⁸. Sequences from chloroplasts of the ancient green algal group Prasinophyceae may be particularly helpful in resolving this question. In addition, Morden and Golden⁶ report a smaller selection of cyanobacterial sequences (7 sequences from 5 taxa compared with 17 RNA sequences). It is still possible that additional *psbA* sequences from cyanobacteria would fall between green chloroplasts and *Prochlorothrix* and thus separate these two groups. It is therefore important to determine more sequences, including the *Cyanophora* cyanelle.

How can the impasse be resolved? I have made suggestions for additional sequences (or temporarily deleting sequences). It is always safer to reconstruct trees by more than one method, wherever

possible using more than a single molecule. One method with one coding sequence seems insufficient to reconstruct unambiguously the whole of evolution. Methods such as convergence, bootstrapping and jackknifing^{12,13} are available to help estimate accuracy.

Where, then, do we stand? The biggest difficulty is in persuading people that collecting sequences is the easy part of any study. The hard part is estimating the accuracy of the results. In some areas of molecular biology it is accepted that, for example, establishing secondary and tertiary structure from sequences is much harder than obtaining the primary sequences. Any measurement, to be scientific, must include an indication of its accuracy. Despite the progress that has been made, reconstruction of evolutionary trees from sequence data must still appear to those in other fields as parascientific. □

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Evolutionary ecology

Diversity of little things

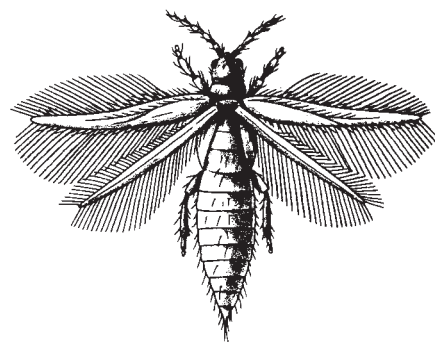
Jon Seger

WE are gigantic animals. If there are 10 million species (probably a conservative estimate^{1,2}), then we are among the largest 0.001 per cent. Songbirds, mice and honeybees seem small to us, but they are almost certainly in the top 1 per cent. The remaining 99 per cent consists mainly of insects and mites with average body lengths of roughly 3 mm^{2,3} (the size at which we cease to notice things even when they land in our soup). An immense diversity of life-histories and reproductive biologies would be expected to occur at these sub-sub-lilliputian scales, simply because there are so many species. In fact, the action appears to be disproportionately concentrated among very small animals. To cite just one of many possible examples, parthenogenesis in its many varieties is overwhelmingly most common among small arthropods^{4,5}. The report by Crespi on page 357 of this issue⁶ describes what is apparently the first known instance of

facultative viviparity, in which individual females can lay eggs, or give birth to live young, or both. This feat is performed by a 5-mm thrips.

Thrips (see figure) belong to the insect order Thysanoptera ('fringed wings'). Most species are small to very small (5-0.5 mm), and most live on plants. The order is thought to be primitively haplodiploid, in the style of Hymenoptera, with haploid males arising from unfertilized eggs (arrhenotoky) and diploid females arising from fertilized eggs. Completely asexual reproduction is known in some species, but most seem to be fully sexual (on the female side). Most species lay eggs, but some are wholly or partially viviparous.

In the species studied by Crespi (*Elaphrothrips tuberculatus*), reproductive mode is associated with sex of offspring: male offspring are produced viviparously and female offspring oviparously. During a given bout of reproduction, a female is



A typical thrips from the order Thysanoptera. Scale bar, 1 mm.

either viviparous or oviparous. But by placing females in fine-mesh bags, under otherwise natural conditions, Crespi demonstrates that many long-lived females switch modes, thereby producing offspring of both sexes.

Because sex of offspring is associated with reproductive mode, and the sex ratio is subject to powerful frequency-dependent selection, roughly equal numbers of females should be found in each mode at any given time. Crespi's study population has two generations per year. In the summer generation there is a slight excess of male-producing, viviparous females, but in the spring there is a large (3:1) excess of female-producing, oviparous females⁶⁻⁸. If this pattern of sex allocation represents an evolutionary equilibrium, then the population probably experiences local mate competition⁹ (differential migration of the sexes) or partial bivoluntinism^{10,11} (differential survival of the sexes between generations), or both.

Adult males guard gravid oviparous females for hours or days at a time, but they usually ignore viviparous females⁷. This behaviour is consistent with the assumption that *E. tuberculatus* is haplodiploid, because male offspring have no fathers under haplodiploidy.

Viviparous females have only about half the fecundity of oviparous females, but newly laid eggs suffer considerable mortality before hatching. In the end, each reproductive mode yields roughly the same number of pupal offspring. Why, then, should male offspring be produced viviparously? One obvious adaptive hypothesis is that males benefit more than do females from entering the world in an advanced state of development. Crespi has previously shown⁷ that because success at mate-guarding is strongly size-dependent, males benefit more from being relatively large than do females.

But if being produced viviparously is good for males, surely it should be good for females as well. Why, then, are female offspring not produced in the same way? A plausible nonadaptive hypothesis is simply that the evolutionary transition to viviparity is easier under parthenogenesis than it is under sexual reproduction,

perhaps because the egg must be fully ovulated before it can be fertilized. Within the arthropods as a whole there seems to be a strong association between viviparity and parthenogenesis (N.A. Moran, personal communication); this pattern is particularly striking in cyclical parthenogens such as aphids and *Daphnia*. Even so, some taxa manage to combine viviparity and sexual reproduction (for example, parasitoid flies that 'larviposit' on their hosts). It will be interesting to see what turns up in thrips as more species are studied in detail.

Large animals easily excite our emotions; small ones probably would if we knew them better. They make up most of the Earth's biotic diversity, in several senses of the word, and they remain largely unnamed and unstudied. This means that many thousands, even millions, of small species may be driven to extinction through habitat destruction¹² before

we even note their existence. But millions more are likely to remain, still the richest part of "life on a little-known planet"¹³. □

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Oxide superconductors

Electron doping tests theories

V. J. Emery

THE discovery of electron superconductivity in cuprate compounds, reported by Tokura *et al.* on page 345 of this issue¹ has significant implications for our understanding of high-temperature superconductors. The charge carriers in previously known cuprate superconductors can be thought of as holes, or missing electrons. Typically an insulating material is rendered metallic by substituting divalent ions for trivalent ones (say, Sr^{2+} for La^{3+}) or by adding oxygen, which enters the compound as O^{2-} : to maintain charge balance, electrons are removed from the (superconducting) copper oxide planes. Tokura *et al.* went in the opposite direction. Starting from Ln_2CuO_4 , where Ln is one of the lanthanides Pr, Nd, or Sm, they replaced Ln^{3+} by Ce^{4+} and removed oxygen by annealing under reducing conditions. In this way electrons are added to the CuO_2 planes, and superconductivity is achieved when the carrier concentration exceeds about 20 per cent per formula unit.

At this early stage, rather little is known about the electronic and magnetic properties of the new materials produced by Tokura *et al.*, although they surely will be studied closely during the next few months. But because the supercurrent is carried by electrons rather than holes and because of the planar copper coordination (see below), the new materials allow new kinds of tests of theories of high temperature superconductivity.

The simplest possibility is that the CuO_2 planes in the insulating parent compounds, Ln_2CuO_4 and La_2CuO_4 turn out to be particle-hole symmetric, that is they behave

in the same way if electrons are added or removed. Indeed this is a property of 'single-band' models²⁻⁴ of the CuO_2 planes, which neglect all the electron states in the material (or energy bands) apart from a partially occupied band bearing the delocalized charge carriers. In some ways this would be the least interesting, but nonetheless important, outcome, replicating properties already observed in existing materials.

An alternative point of view⁵ is that the Coulomb interactions between the charge carriers are so strong that other bands cannot be ignored. In the parent compounds the $\text{O}(2p)$ levels are filled and the holes are in $\text{Cu}(3d)$ states, giving Cu^{2+} ions with a spin that is responsible for the observed magnetic behaviour. When holes are added by doping they go into $(2p)$ orbitals because the strong Coulomb repulsion makes Cu^{3+} extremely unfavourable energetically. Because the $\text{O}(2p)$ orbitals become partially filled, the added holes can form a conduction band which ultimately leads to superconductivity. Of course the situation is not so extreme, there is some hybridization (mixing of states), but to talk of localized moments on copper and mobile holes on oxygen is a convenient 'zeroth order' way of speaking.

There is much evidence^{6,7} in favour of this picture mainly from X-ray, electron and ultraviolet spectroscopy as well as from neutron-scattering experiments. Indeed, the outstanding issue is not so much whether the holes are on oxygen but rather which of the possible $2p$ states are

populated. Electron doping would give a different result, however, because the $\text{O}(2p)$ level is filled in the parent compound, and the electrons must go onto copper sites to produce Cu^+ . This conclusion could be verified by X-ray absorption near-edge spectroscopy⁸ for example. It is clear that the T^* structure of the new materials¹ (in which each copper ion sits in a square plane of oxygen ions) is electrostatically favourable for electron doping onto copper: unlike other structures, there are no apical O^{2-} ions sitting directly above or below the copper sites repelling the excess charge.

Naively, the closed-shell Cu^+ ions behave like vacancies (missing spins) in the array of local Cu^{2+} moments which are magnetically coupled with a strength J . The vacancies are mobile because an electron can hop with amplitude t from Cu^+ onto a neighbouring Cu^{2+} . This is formally identical to the so-called t - J model⁹ which may be derived from the single-band model²⁻⁴ in the limit of strong interactions, and has been widely used to study magnetic mechanisms of superconductivity. In this way, the simplest versions of the two models might coincide for electron doping. At the same time, there is an ongoing debate about whether, for some purposes, the same may be true for hole doping. Specifically, for low-energy properties, such as magnetism, the mechanism of superconductivity and parameters of the superconducting state, does a hole on oxygen behave in the same way as a missing spin^{9,10}? If so, there would be a limited and more elaborate form of particle-hole symmetry. The new materials may shed some experimental light on this important question.

A study of magnetic properties will be particularly interesting in this regard. It is already known from neutron-scattering experiments by Torardi *et al.* (D.E. Cox, personal communication) on Pr_2CuO_4 and muon-spin-rotation ones by Luke *et al.* (Y. Uemura, personal communication) on Pr_2CuO_4 , Nd_2CuO_4 and Sm_2CuO_4 that the end members have magnetic order. For Pr_2CuO_4 , the ordering temperature is about 260 K and the magnetic structure is similar to that of La_2CuO_4 , with an ordered moment of about 0.5 Bohr magnetons per copper atom (Cox). An extension of these studies to the doped materials will enable us to contrast the effects of holes on oxygen and electrons on copper

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as the magnetic order gives way to superconductivity.

Of course this naive description of electron doping is not the whole story. A Cu^+ ion will polarize its environment, giving rise to some density of holes on the surrounding oxygen sites. Just as for hole doping, the charge carriers are complex and extended entities and there are more routes to superconductivity than the simple t - J model allows. Many proposed

mechanisms will require rethinking. Those relying on a displacement of the position or the charge of apical oxygens or the associated splitting of the $\text{Cu}(3d)$ levels are clearly ruled out. Others emphasizing charge transfer between copper and oxygen must adapt to the new location of the charge carriers. $\square$

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Astronomy

Mass loss from Wolf-Rayet stars

Roger J. Tayler

WOLF-RAYET stars are very massive stars which are undergoing very rapid mass loss, typically several times 10^{-5} solar masses ($M_{\odot}$) per year¹. Although most investigators believe that the mass loss is primarily driven by the pressure of the star's radiation, the momentum being communicated to the flow by very strong absorption in selected spectral lines, there is not complete agreement on this point. In a recent paper, Moffat *et al.*² report observations of five Wolf-Rayet stars in which the mass loss has an irregular blob-like structure and they believe that this property might help to distinguish between various proposed mass-loss mechanisms.

It has become clear during the past 20 years that a very wide variety of stars experience mass loss at rates ranging from the solar wind of a few times $10^{-14} M_{\odot} \text{ yr}^{-1}$, or less, to the dramatic loss of many $M_{\odot}$ in the explosion of a supernova. In most cases the mass loss becomes apparent through the existence of very broad emission spectral lines which can be understood only in terms of mass leaving the star; in some cases direct observations are ambiguous as regards infall or outflow but there are usually other features which

resolve the ambiguity. The most simple theoretical discussion of mass loss assumes that it is steady and spherically symmetric and such a discussion gives a first qualitative understanding of what is happening. It is, however, by no means certain that mass loss has this property.

For example, if the star is rapidly rotating or possesses a significant magnetic field, it will not be spherical and it is therefore unlikely that any mass loss will have spherical symmetry. If the mass loss is initiated by pulsation of the star, there will be time variations. Finally, a spherically symmetrical outflow might be thermally unstable, leading to the formation of cool dense blobs in a hotter diffuse flow. Evidence for such departures from the simple model can be found by studying the detailed profiles of the spectral lines which provide evidence of mass loss.

In the case of stars with low surface temperatures such as the Sun, the mass loss is strongly influenced by, if not determined by, the structure of the magnetic field at and above the star's surface. The field is maintained by a dynamo process in the star's outer convection zone and its strength seems to depend on the

rotation speed of the star. The field structure is complex with some magnetic field lines forming closed magnetic loops within a few stellar radii and with others extending to large distances from the star. It is not surprising that in this case the mass loss is not spherically symmetrical. It is concentrated along the open field lines, while the energy input at the base of the closed loops raises the confined gas to much higher temperatures. Interestingly, in the case of one cool star, AB Doradus, Robinson and Collier-Cameron³ have shown that the gas confined by the loops has apparently suffered thermal instability so that cool clumps of matter are embedded in the hot gas.

Moffat *et al.*² now show, by a study of emission lines of helium in five hot Wolf-Rayet stars, that their mass outflow must contain blobs of material. What they have observed are bumps in the spectral line profiles, which have the property that those starting redshifted relative to the line centre become more redshifted with time, while those starting blueshifted become more blueshifted with time. Their conclusion is that they are seeing blobs of material which are being accelerated outward, some on the near side of the star and some on the far side. Their study of the time of appearance of the blobs indicates that they cannot be related to any fully predictable property of the star, such as rotation or magnetic field structure or a regular pulsation. The blobs appear to arise randomly in starting time and in direction outward in the flow and this can probably be understood in terms of a stochastic instability of a radiatively driven wind as was proposed by Rybicki⁴.

In his discussion, Rybicki⁴ developed work by other authors on various possible instabilities in outflowing winds. The sensitive velocity dependence of radiative driving due to absorption in a spectral line is a particularly important source of instability. An element of material moving faster than neighbouring elements Doppler shifts the spectral line to a wavelength which has not yet suffered absorption and this leads to stronger driving and instability. Because of the outward motion such instabilities are rapidly advected away and they cannot form a coherent pattern around the star. This appears to accord with the observations of Moffat *et al.*², although further work will be needed to determine whether quantitative agreement between theory and observation exists. $\square$

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REASONS

Agricultural genetics

Moving in on plant genes

Robert Shields

VIRTUALLY nothing is known about the protein products of the genes controlling morphology, dormancy, photoperiodicity and disease resistance in plants. Two techniques being used to try to clone these genes are transposon tagging and gene mapping. Transposons (mobile pieces of DNA) can generate different phenotypes which should allow the genes governing the phenotype to be isolated; and mapping should allow specific genes to be targeted. Although tagging is the more elegant technique, a new report¹ by Young *et al.* indicates that mapping may be the first method by which one of these genes is uncovered.

Transposons can be used as simultaneous mutagens and gene tags. Several plant genes (mainly those involved in pigment biosynthesis) have been cloned in this way from maize or snapdragon. The discovery² that the transposon Ac in maize could be transferred to other plants, where it is moved from its original site of insertion to a new locus, was therefore met with considerable excitement. Self-fertilization of the resulting plant will give the tagged locus in a homozygous state and simultaneously reveal a new phenotype. But things may not be that straightforward. In its native host, Ac tends to excise and then reinsert at a closely linked site on the same chromosome³. If this is true in the heterologous host, it will be necessary to produce plant lines with transposons inserted close to target genes before tagging can begin. In other organisms such as the fruitfly *Drosophila*, where transposons act as mutagens, specific loci are mutated at vastly different rates as the elements show strong site-specificity for insertion⁴. So, although it may be possible to isolate plant genes by transposon tagging, there may be problems in approaching specific loci by this method.

In several plant species, genes have

been assigned to positions in genetic linkage groups which in a few plants can be correlated with cytologically identifiable chromosomes. The resolution of many of these linkage maps is poor and several laboratories are attempting to improve them by constructing maps based on restriction fragment length polymorphisms (RFLPs). These maps have a use as a practical breeding aid, for if an RFLP marker shows close linkage with a desirable gene then progeny in breeding experiments can be rapidly screened for its presence without the necessity for large-scale field trials. A secondary use of such maps is to use them as starting points for attempts to isolate the linked gene.

The first requirement is to position the target gene accurately on the RFLP

and the RFLP map is limited by the frequency of allelic variation in the organism examined (which determines the likelihood of finding RFLPs) and the number of cases that can be examined where the target gene and closely linked RFLPs are segregating (which gives the map position). In practical terms 1 centimorgan (1 per cent recombination) is close to the limit of resolution achievable without examining a prohibitively large number of crosses unless near-isogenic lines can be used to give closer linkage. The table shows that for plants such as *Arabidopsis*, which has a very small genome, 1 centimorgan represents 139 kilobases, thus chromosome walking from an RFLP marker to a genetic loci is just within the bounds of possibility, given a lot of effort. Doubtless other genes will be isolated in this way from *Arabidopsis* and will then be used as probes to find homologous sequences in other plants. But it seems unlikely that genes governing many complicated plant functions (such as disease resistance) would be similar

The resolution gap in plants

Species	Genome size (kilobase pairs)	Map length (centimorgan)	Kilobase pairs equivalent to 1 centimorgan	Ref.
<i>Arabidopsis</i>	7×10^4	501	139	10
Tomato	7.15×10^5	1,400	510	1
Human	3×10^6	2,708	1,108	11
Maize	3×10^6	~1,400	~2,140	12

Map lengths are based on published RFLP data. The conversion of centimorgans to kilobase pairs is an average value as recombination frequency varies in different chromosome regions.

linkage map and if necessary find new RFLPs which map closer to the gene. Young *et al.* have now elegantly demonstrated¹ the combined use of RFLPs and near-isogenic lines to find markers very close to a plant disease-resistance gene. Plant breeders often cross a cultivated variety with an alien species carrying a desired gene and then backcross to the cultivated variety (the recurrent parent) while selecting to maintain the required trait. After a series of such crosses, the genome of the selected plants will consist almost entirely of the genome of the recurrent parent with a small segment surrounding the target gene which comes from the alien genome. By examining RFLP differences between the original variety and the new near-isogenic line, it is possible to identify RFLP markers lying very close to the introgressed gene. Young *et al.* find markers lying 0.4 ± 0.4 centimorgans ($= 2 \times 10^5$ base pairs in tomato) of the *Tm2a* gene (a dominant gene giving resistance to tobacco mosaic virus by restricting cell-to-cell spread) which had been introgressed into the cultivated tomato (*Lycopersicon esculentum*) from the wild variety *L. peruvianum*. Near-isogenic lines should be extremely valuable for rapidly assigning introgressed genes to their map position.

The correlation between the genetic

between *Arabidopsis* and economically important crop plants.

For such plants a 'resolution gap' exists between the genetic and the physical map which must be bridged if their genes are to be approached from linked RFLP probes. A similar (although less daunting; see table) resolution gap in the human genome has spurred technical developments such as pulsed-field gel electrophoresis⁵ and cloning in yeast artificial chromosomes to handle and map very large pieces of DNA^{6,7} as well as the use of 'jumping' and 'linking' libraries to overcome the necessity of walking everywhere in the genome^{8,9}. Once the region surrounding the target gene is physically mapped, ways must be found to identify the gene itself. □

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100 years ago

WITH reference to A. C. Haddon's account of the use of the *Remora* or sucker fish by the natives of Torres Straits in fishing for turtles (*Nature*, 17 January, p.285, 1889), I may call attention to the paper on this subject read by Frederick Holmwood, C. B., late H.B.M. Consul at Zanzibar, before the Zoological Society of London, on 17 June 1881, which Haddon does not seem to be acquainted with. Holmwood has fully described the mode of the use of the *Remora* by the native fisherman of Zanzibar in catching turtles and fishes. It is curious to find a somewhat similar method of employing the *Remora* practised by the islanders of Torres Straits.

P. L. SCLATER

From *Nature* **39**, 295; 24 January 1889.

Hexagonal polar current on Saturn

THE remarkable hexagonal feature surrounding Saturn's north pole apparent in the figure was discovered by D. A. Godfrey (*Icarus* 76, 335–356; 1988) while making polar projections of Voyager images. The outermost circle is at about 65° latitude and represents the edge of a zonally symmetric current in Saturn's atmosphere. At the time of the spacecraft fly-by in 1981 the northern hemisphere was entering spring and the polar region was sufficiently illuminated to show features, although great care was necessary to remove shading due to twilight effects. The mosaic figure, one of six presented (each showing the hexagon), is made up of four images taken of the sun-lit hemisphere as the planet rotated. Because Voyager's orbit kept it close to Saturn's equator, Godfrey had to reproject the foreshortened images of the north pole to produce the view shown, looking down the planetary axis. The morphology of the clouds does not change a great deal during one 10-hour rotation period, so that the mosaic is not very much different from an instantaneous snapshot. The four 'spokes' are boundaries between images, and the streaked appearance near the pole is an artefact of the projection.

Small differences between sequential mosaics can be used to determine the drift rate of clouds, and it is here that surprises emerge. The hexagonal feature is an atmospheric jet stream whose position and configuration remain fixed, to within a few metres per second, but the current in the jet exceeds 100 m s^{-1} . In this context, 'fixed' means stationary in latitude at about 76°, and in longitude relative to the periodicities which appear in radio emissions from the planet, presumably linked to the magnetic field which in turn is anchored to the deep core of the planet. Thus this is a jet stream which follows a twisting yet stationary path, rather than meandering, as is the familiar case in Earth's atmosphere and oceans.

Godfrey discusses the possibility of a direct connection between the hexagonal structure and the magnetic field, but concludes that it is improbable. The clouds are ammonia crystals at a level where the atmospheric pressure is about half an atmosphere and the temperature is about 120 K, well within the 'troposphere' (lower atmosphere) rather than the ionosphere. The conductivity is low and

magnetohydrodynamic effects should not exist. Also unlikely is a direct connection between the radio emissions and the planetary weather, as these emissions do not have the character of lightning bursts. The simplest idea is that the magnetic field



D. A. Godfrey

second, although it is composed of (and resides in) currents whose speeds reach 100 m s^{-1} . Smaller spots on Jupiter also tend to be slowly moving. T. E. Dowling and A. P. Ingersoll (*J. Atmos. Sci.* 45, 1380–1396; 1988) have shown that the circulation patterns near and within these spots can be used to infer the nature of

flows at deeper levels, although they do not point to any particular reason for very slow motion of the spots. And there are slowly moving large-scale thermal features, discovered in the infrared by J. A. Magalhães *et al.* (to be published in next week's *Nature*) located at low latitudes on Jupiter, right where the broad equatorial current is also situated. The current is apparently perturbed by some influence that is nearly stationary.

All of these features could be providing information about deeper regions. Jupiter and Saturn have only small solid cores, unlikely to have any influence on surface motions. The vast bulk of these planets is fluid hydrogen and helium in roughly cosmic abundance. Heat emerges from the interiors in large quantities,

comparable to the solar heating. The nature of the fluid motions which carry this heat to the surface has long perplexed astronomers and those studying convection. We may at last be seeing evidence, albeit indirect, for the morphology and flow speeds associated with this convection.

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Palaeontology

Fossil reptiles from ancient caves

Michael J. Benton

ONE of the problems of studying terrestrial fossil sites is that large plants and animals seem to be commoner than small ones, whereas the opposite pertains in nature. This could be because organisms were on average larger in the past; or because small ones are less likely to be preserved. The latter explanation seems to be the case as sites have been found, although only occasionally, which contain abundant small fossils. One such is the fossilized cave systems of south Wales and Bristol in the United Kingdom. In a new paper, N.C. Fraser¹ reports the discovery of small

animals from an age where most sites reveal dinosaurs and other large animals.

The abundant small beasts of the Bristol–South Wales region offer a fascinating glimpse of life on land beneath the feet of the first dinosaurs. Despite their abundance and the good quality of preservation, the fauna is not perfectly fossilized. The skeletons are found at the bottom of fissures eroded into the ancient limestone hills. As the openings into the fissures are often small, the larger animals have effectively been filtered out. Nevertheless, the fissure animals provide unique

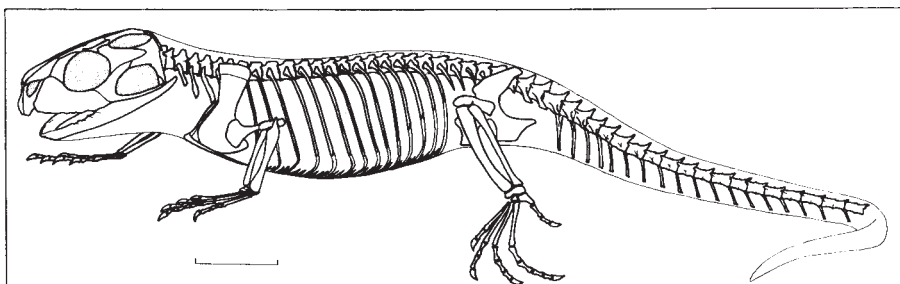


Fig. 1 Composite restoration of the entire skeleton of *C. hudsoni*. Bar, 2 cm. (From ref. 1.)

insights into the lives of the more abundant animals at this time of major faunal turnover. Some of the fissures were under water at the time of fossil formation.

The fossiliferous fissure deposits are dated as Late Triassic to Early Jurassic (about 220–200 million years old), the time during which the dinosaurs became established, and during which most 'modern' land vertebrates arose: mammals, turtles, crocodilians and lepidosaurs. These smaller 'modern' vertebrates are best represented in the fissures and they have yielded significant new phylogenetic information.

Fraser's find¹ of the spenodontid *Clevosaurus hudsoni*² allows him to perform a detailed analysis of this animal and of its relationship with other organisms. *Clevosaurus*, a small 250-mm-long reptile, is superficially like a modern lizard, with a slender body, long limbs and (probably) a long tail (Fig. 1). The teeth are chisel-like and, unusually for a vertebrate, fused firmly to the bone of the jaws (Fig. 2). The teeth and jaw bones of adult specimens of *Clevosaurus* show signs of heavy wear, and the jaw action was a shearing one, rather like the action of a well-adjusted pair of scissors. *Clevosaurus* probably fed on hard-skinned invertebrates such as centipedes, insects and

snails. It may also have been a facultative herbivore, raking plant food together with its beak-like front teeth, and chopping it further back in the mouth. Young *Clevosaurus* specimens have small, sharp teeth which may have been used for eating soft-bodied invertebrates such as worms.

Fraser¹ distinguishes two or more species in his material of *Clevosaurus*. The material is mainly disarticulated, and he examined more than 1,000 separate bones. Fortunately, he found a few more complete specimens which helped in the identification of isolated elements. The second *Clevosaurus* species, *C. minor*, is much smaller than *C. hudsoni*, reaching an estimated total length of 150–200 mm. They have adult patterns of tooth wear, so are unlikely to be juveniles of *C. hudsoni*. *C. minor* is found at different sites from *C. hudsoni*, so they are probably independent breeding species.

Other associated vertebrates from the Triassic fissures³ include a gliding reptile, *Kuehneosaurus*; the early crocodilian *Terrestrisuchus*⁴; the dinosaur *Thecodontosaurus*⁵; the very early mammal *Kuehneotherium*⁶; various unidentified archosaurs⁷; and up to ten species of spenodontid^{1,8–10}. The Jurassic fissures contain a rather different fauna, consisting of the mammal *Eozostrodon*, the tritylodont *Oligokyphus*, the lepidosaur *Gephyrosaurus* and fish teeth⁸.

The spenodontids, such as *Clevosaurus* and its kin from the fissures, are of great phylogenetic importance. They are broadly similar to the living tuatara *Sphenodon* from New Zealand, a so-called living fossil. The fact that a complex fauna of up to ten spenodontids co-existed in the south-west of Britain 220–210 million years ago suggests that the group was once as flourishing as the lizards are today¹⁰. □

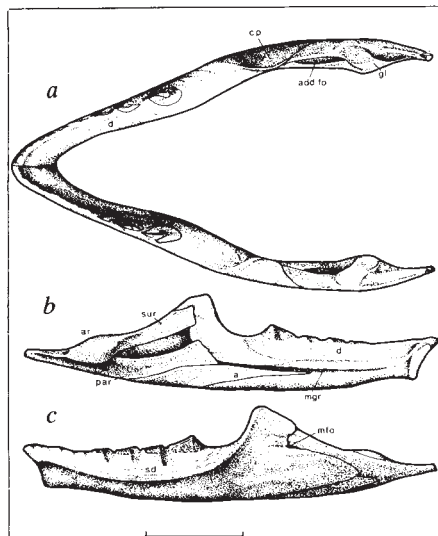


Fig. 2 Composite restoration of the mandible of *C. hudsoni* in: a, dorsal; b, medial; c, lateral views. Scale bar, 1 cm. (From ref. 1.)

Daedalus

If I had a hammer

THE hammer is one of the most basic of implements. It exploits a simple newtonian principle: the deceleration of its head imposes a force on the object it strikes. In the hands of a skilled craftsman, it can crack a brick precisely in two, or make a controlled adjustment of 0.01 millimetres.

In the hands of unskilled amateurs, however, it produces a vast global total of bent nails, bruised thumbs, dented woodwork and smashed panes of glass. Daedalus is therefore updating this palaeolithic invention with the most advanced electronics. He has been inspired by the modern thyristor photographic flash unit. This integrates the light reflected off the scene and, when enough has been received for a good exposure, quenches the flash. It all happens in microseconds. In the same way, Daedalus's electronic hammer carries an accelerometer which integrates the momentum received by the target during the impact. When this exactly reaches a pre-set value, the impact is 'switched off'. A fast-acting trigger mechanism snatches back the hammer head and dumps the surplus momentum back into the handle and the user's arm.

This wonderful tool not only tames and directs the enthusiasm of the user: it educates him too. Set for a delicate task (such as tapping a ceramic tile into place on its mortar) it will transmute a wild swipe into a light and precise blow. But the excess momentum, returned to the user's arm as an unexpected 'kick', warns him that his blow was too heavy. His next swipe will not be so wild. Soon he will have a sound intuitive feeling for the exact use of the hammer in this task. Professionals and amateurs alike will welcome the electronic hammer: in all fields of engineering and domestic construction it will teach the subtle kinetic intuition of the true craftsman. Indeed, electronically controlled impact may become the preferred way of carrying out all sorts of tasks now conducted manually, from removing a stopper to advancing a pawn or shaping a cake. Daedalus is devising a 'personal electronic hammer' to fit in the pocket like a pen. The user will turn to it for any manual task as automatically as he reaches for a pen to write with, or a calculator to do arithmetic.

But the best strategy for developing a new invention is to launch a high-value product first, on whose profits the mass market can be attacked. So Daedalus's first commercial product will improve the most valuable impacts of them all — those of international golf. His electronic golf clubs, bringing calibrated exactitude to this most obsessive and unforgiving of games, should sell for tens of thousands of pounds.

David Jones

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Setting radiation standards

SIR—The setting of standards of protection against ionizing radiation, already controversial, is being confused by the difficulty of specifying clearly the significance of the risk to an exposed individual. 'Risk' is used here to mean the probability of a deleterious event. The term 'detriment' can be used to combine the probability and the severity of that event.

For many years, the fatal risk to an individual has been presented as the probability of attributable death with no allowance for competing causes of death. The effect of this allowance is known, but has not been used explicitly in setting standards. The effect of latency on the degree of detriment has also been neglected, although a death in later years is less detrimental than an earlier one.

More recently, in its 1988 report to the UN General Assembly¹, the United Nations Scientific Committee on the Effects of Atomic Radiations (UNSCEAR) has addressed both these issues. The allowance for competing causes of death is explicitly included in the risk estimates, and the effect of latency on the detriment is indicated by estimating the average years of life lost. Information on the distribution of risk with time after exposure is still incomplete because many members of the exposed populations being studied are still alive. UNSCEAR offers two risk-projection models, one with the risk constant (after a latent period) — an additive model — and the other with the risk increasing with time as does the risk of 'natural' cancer — a multiplicative model. The latter predicts about twice as many deaths as the former

with no more years of lost life. Unfortunately, most commentators on this topic have stressed the increase in fatal risk without considering the decrease in years lost per attributable death.

There is a further weakness in using risk alone. The total risk of death cannot be increased; it is unity. Radiation can change the time and cause of death, not the overall probability.

The risk to the individual is thus a poor measure of the consequences of exposure to ionizing radiation. We need a multi-attribute approach to take account of the time and nature of a death in addition to its probability. This would also make it easier to include explicitly the non-fatal effects such as curable cancer and hereditary defects. It is to be hoped that those responsible for setting standards, particularly dose limits, will not rush into changes based only on the simple concept of risk. As both the International Commission on Radiological Protection² and the National Radiological Protection Board³ have indicated, the most appropriate immediate reaction is to increase the attention paid to the need to keep all exposures as low as can reasonably be achieved. Quantitative changes in standards should be a more measured response.

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X-chromosome reactivation and ageing

SIR—At first sight it seems that the results of Migeon *et al.*¹ on the low reactivation frequency of the human X-linked hypoxanthine phosphoribosyl transferase (HPRT) gene do not confirm the earlier finding of the strong age-related reactivation of the mouse X-linked ornithine carbamoyl transferase gene (OCT)². Whereas the spontaneous reactivation of the silent OCT gene was increased 50-fold in mice older than one year, the reactivation of the silent human HPRT gene in female Lesch-Nyhan heterozygotes is rare at any age.

In studies of the process of ageing, one of the main challenges is to understand how similar physiological or biochemical events occur at very different rates in species with different lifespans. For example the crosslinking of collagen is known to increase with age, but although the chemistry is the same, the rates of reaction are very different in different species³. The same is true for the accumu-

lation of the age pigment lipofuscin, the denaturation of lens crystallin and other 'biomarkers' of ageing, as well as many pathologies, including the appearance of neoplasms. It is well known that mouse cells transform to malignant cells readily in culture, whereas human cells are very refractory to this transformation. This makes biological sense, because on a per cell basis, small short-lived animals have a far higher incidence of cancer than large long-lived ones.

It is therefore reasonable to argue that the age-related reactivation of a silent X-linked gene would be many times higher in mouse cells than in human cells. Another way of saying the same thing is that the controls of cellular metabolism, or cellular homeostasis, must be very much tighter in long-lived animals than in short-lived ones, and this would of course include the silencing of gene expression by DNA methylation⁴. It is already known that the maintenance of cytosine methylation in

DNA in primary diploid cells is much more effective in human than in mouse cells, and that hamster cells have an intermediate level of maintenance⁵.

Two of the results reported by Migeon *et al.* together indicate that there may be a low loss of methylation in normal human cells with ageing.

First, there is a slight increase with age in the proportion of cells with active HPRT, which is an insensitive assay, and the results are of marginal statistical significance ($P=0.08$).

Second, using a much more sensitive assay, it is shown that the silent HPRT gene can be more easily reactivated by the demethylating agent deoxyazacytidine in cells from old individuals than in cells from young ones ($P<0.005$).

These results would be expected if the methylation of many sites in the CpG-rich island of the human HPRT gene declines slowly with age, but the overall silencing mechanism is fairly efficiently maintained. However, a gradual loss of methylation would increase the probability of reactivation by a demethylating agent. If this interpretation is correct, then similar experiments carried out with female 'Lesch-Nyhan' heterozygous mice^{6,7}, should show a significant age-related reactivation of HPRT. Such a result would reinforce the view that cellular homeostasis is much better maintained in long-lived species than in short-lived ones^{4,8}.

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Misidentified cell

SIR—Because the cell line TE671 is said to originate from an early culture of a human medulloblastoma¹, it has been widely used to investigate this tumour type and as a model for primitive neural cells in general. And because the cell line can be infected by the AIDS virus HIV² (in a way that cannot be blocked by soluble CD4, as reported on page 368 of this issue³), TE671 could provide an ostensibly useful *in vitro* model of HIV infection of the brain. Our evidence, however, suggests that TE671 is not a neural cell but a muscle cell.

We note that TE671 cells have a number of features in common with the rhabdomyosarcoma cell line RD. They have several of the phenotypic characteristics of striated muscle cells, including the presence of desmin, myoglobin⁴ and the

muscle-type acetylcholine receptor⁵. We have found that both cell lines contain an *N-ras* gene that is activated by a point mutation at the third base of codon 61 and, in new cell stocks obtained directly from the American Type Culture Collection, that the majority of marker chromosomes are common to the cell lines. Furthermore, DNA fingerprinting with locus-specific and core probes fails to reveal differences between the two lines.

Both cell lines were derived in the same laboratory but RD was described⁶ and submitted to the ATCC before the first report of TE671. When considered together these observations indicate that TE671 is most probably a subline of the rhabdomyosarcoma cell line RD.

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Spectral peaks

SIR—Lutz and Watson¹ discuss the frequency spectrum of the Harland *et al.*² geomagnetic reversal record of the past 165 million years. This spectrum has been interpreted as showing a 30-million-year short-term oscillation superimposed on an aperiodic long-term variation. Lutz and Watson argue that the long-term component, $\lambda_L(t)$, dominates the frequency spectrum for periods P up to 40 million years. Here I show that the spectrum in this range is essentially determined by the strong boundary discontinuities introduced by performing a Fourier transform $\lambda_L(t)$ over a finite interval (τ_1, τ_2) ; that is, these discontinuities dominate over any time variation of $\lambda_L(t)$ between τ_1 and τ_2 .

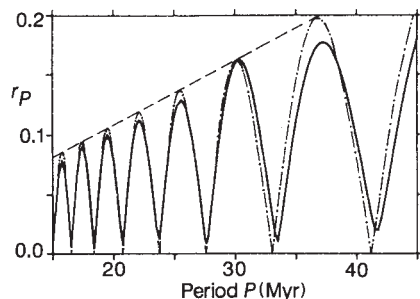
The influence of discontinuities becomes apparent when the limits of the Fourier transform are extended to $(-\infty, +\infty)$. This leaves all integrals unchanged, provided that the 'extended' function $\lambda'_L(t)$ has the form

$$\lambda'_L(t) = \begin{cases} \lambda_L(t) & \text{for } \tau_1 \leq t \leq \tau_2 \\ 0 & \text{otherwise} \end{cases} \quad (1)$$

As $\lambda_L(t)$ is largest at the boundaries, $\lambda'_L(t)$ exhibits large discontinuities there. The dominance of these discontinuities can be demonstrated by considering a simplified trial function

$$\lambda'_L(t) = \begin{cases} \lambda_d = \text{constant} & \text{for } -T/2 \leq t \leq T/2 \\ 0 & \text{otherwise} \end{cases} \quad (2)$$

which retains the two boundary discontinuities (of size λ_d , taken to have the value 5 Myr^{-1} , and separation $T = \tau_2 - \tau_1$) but carries no information on variations in between. The spectrum of this function,



Fourier spectra of two long-term reversal functions plotted against period $P = 2\pi/\omega$. $R_p^1(\tau_1, \tau_2)$, from ref. 1, is shown as a solid curve, and $R_p^0(T)$ (equation (3)) as a dash-dotted line. The straight dashed line is the exact asymptotic envelope for $P < T$ to both spectra.

tinuities (of size λ_d , taken to have the value 5 Myr^{-1} , and separation $T = \tau_2 - \tau_1$) but carries no information on variations in between. The spectrum of this function,

$$R_p^0(T) = N^{-1} \lambda_d (P/\pi) |\sin(\pi T/P)| \quad (3)$$

is compared in the figure with that of $\lambda'_L(t)$ obtained by Lutz and Watson¹. (Here N is the total number of reversals.) We interpret the close correspondence in shape, position and peak heights as verification of our initial claim. The straight dashed line denotes the exact small- P asymptotic envelope for any function $\lambda(t)$ that has the same discontinuities as $\lambda'_L(t)$ and $\lambda'_L(t)$. The positions of the maxima of $R_p^0(T)$ are given by $P_n \approx T/(n+1/2)$ for integers $n \geq 0$; for all T , they agree well with the values from the numerical Fourier transform of $\lambda_L(t)$ given in Table 1 of ref. 1, for which τ_1 was varied in discrete steps. Therefore, the grouping together there of peak positions with more or less constant period is arbitrary. When $\tau_2 - \tau_1$ decreases continuously, the peak positions do so too.

The influence of short-term oscillations would thus be seen only as noticeable deviations of the peak heights from those of $R_p^0(T)$, that is, from the envelope of $R_p(\tau_1, \tau_2)$. Using a Poisson process, Lutz and Watson¹ show that the long-term component alone may generate fluctuations in the peak heights that are large enough to account for the spectrum of the total reversal function $\lambda(t)$.

Thus, for periods $\leq 40 \text{ Myr}$, the spectrum $R_p(\tau_1, \tau_2)$ of the total reversal function is determined mainly by the strong discontinuities in $\lambda_L(t)$ at τ_1 and τ_2 , and hides any information on short-term variations. These can be reliably detected in the spectrum only if the discontinuous long-term variation can be subtracted before the Fourier transform operation.

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RNP in maize protein

SIR—We wish to point out an interesting feature in the sequence of the abscisic acid-induced glycine-rich protein (AAIP) of maize recently reported by Gómez *et al.*¹. The protein contains a sequence (see below) which conforms perfectly to the RNP consensus sequence^{2–4} that has been found so far in heterogenous nuclear RNA-, messenger RNA-, pre-ribosomal RNA-, and small-nuclear RNA-binding proteins.

Maize AAIP:

...⁴⁹ Arg Gly Phe Gly Phe Val Thr Phe⁵⁶...

RNP consensus sequence:

... Lys Gly Phe Gly Phe Val Thr Phe...
Arg Tyr Ala Tyr X Tyr

This strongly suggests that AAIP is a single-stranded nucleic-acid binding protein, most probably an RNA-binding protein (RNP). Several other features of the AAIP sequence also resemble those found in some of these proteins. The protein appears to contain two distinct domains: an amino-terminal domain (about 90 amino acids) that contains the RNP consensus sequence, and a carboxy-terminal glycine-rich domain. The amino-terminal domain is the putative RNA-binding domain⁴. There are extensive similarities in the general character of the amino-terminal domain with that of the putative RNA-binding domain of several RNP proteins⁴. A glycine-rich domain is also a feature of some RNP proteins (for example, the hnRNP protein A1 (ref. 5), and the nucleolar protein nucleolin⁶). AAIP may be the first plant protein described so far to contain an RNP consensus sequence. The predicted property of AAIP as an RNA-binding protein can be readily tested, for example, by chromatography on small-subunit DNA columns, on ribohomopolymer columns, and by photochemical crosslinking to RNA.

Absciscic acid is a hormone that appears to modulate the response of plants to adverse conditions, and it seems to also play a role in embryogenesis. Determining if AAIP is indeed an RNA-binding protein, and if so, what RNA it binds to should be important for understanding the mechanism of these important processes.

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Prized developmentalist

Tim Hunt

The Heritage of Experimental Embryology: Hans Spemann and the Organizer. By Viktor Hamburger. Oxford University Press: 1988. Pp. 196. £22.50, \$29.95.

VIKTOR Hamburger, as old as the century, was a graduate student in Hans Spemann's laboratory in Freiburg. He was an exact contemporary of Hilde Mangold (*née* Proescholdt), who performed the famous 'organizer experiment' (see figure) that clinched Spemann's reputation and won him the only Nobel prize ever awarded for developmental biology. Over 30 years later, in St Louis, Missouri, Hamburger was head of the laboratory in which nerve growth factor and epidermal growth factor were discovered by Rita Levi-Montalcini and Stanley Cohen, work that in turn won them a Nobel prize. It is clear from Levi-Montalcini's account of this period that Hamburger was all that a boss should ever be, encouraging, supportive, insightful and critical, and only his perfect modesty explains why he is not as celebrated as his erstwhile mentor or his own pupils.

Hamburger's account of Spemann and the organizer is a masterpiece. "No subject in embryology has been so misrepresented and misunderstood as the properties of the organizer" according to Jonathan Slack¹, but Hamburger does a superb job of explaining the experiments, setting them in historical context and tracing their subsequent experimental and analytical history. Anyone with the faintest interest in developmental biology should read this book. It is an eye-opening inspiration.

The purely scientific account is enlivened by biographical sketches of the protagonists, done with graceful affection and respect. An appendix describes Hilde Mangold and brilliantly evokes student life in Freiburg in the early 1920s. We discover that the PhD students actually had to know some philosophy, attending lectures by Husserl and Heidegger. The political climate was violent, the inflation appalling, yet the arts flourished and the mountains frequently called the young away to relax; no wonder such great work was done. And although they "cared more for food for thought than nourishment for our bodies", the dreadful "tur-nip winter" put them off that vegetable for life.

Hamburger was close to Hilde Mangold, who alas died in 1924 from burns sustained when a petrol heater exploded at home. She had not been pleased when Spemann insisted on putting his name on her paper (and first at that); but according to Hamburger, the design and conception of

the experiments were Spemann's and Mangold was the fortunate student assigned to carry them out. She died before her work became widely appreciated.

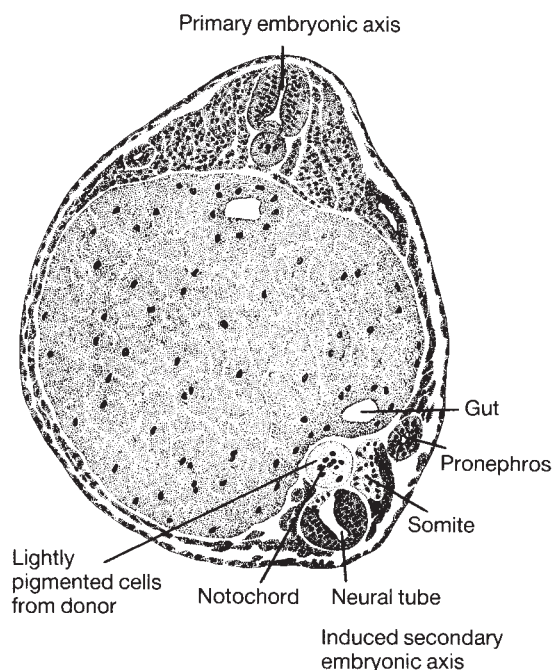
Hamburger analyses these classical experiments and ideas in developmental biology as clearly as I have ever seen it done, and traces Spemann's scientific legacy almost to the present day. He points out errors, ambiguities and misconceptions as well as describing the technical problems that beset the early workers. Spemann, it turns out, did not appreciate the importance of the ionic composition of the medium for the survival of his embryos (at one time he had to send back to Würzburg for water to make things work; calcium was probably the missing ingredient). It was Johannes Holtfreter who discovered the importance of this and of the need for scrupulous sterility, which allowed him to perform much more sophisticated experiments. Nowadays, antibiotics assure the embryos' well-being, and advances in reproductive physiology mean that experiments do not have to attend on the natural breeding season of the animals.

Nevertheless, Spemann was a great

craftsman. His use of baby's hair for the 1904 constriction experiments is legendary, and he invented all sorts of other useful tools for cutting and transplanting bits of the tiny embryos. Moreover, his analytical acumen matched his experimental powers, and though he confessed to a preference for "psychical" rather than physical metaphors in the analysis of his data², he was never carried away by more abstract theory of the kind that led Driesch ultimately to espouse vitalism. In his imagination Spemann was at one with the objects of his study, which allowed him and his followers to extract the maximum of clear information from their transplantation experiments. As Walther Vogt, the great fate-mapper, explained at Spemann's sixtieth-birthday celebration: "The result is not sought by theorizing, thus passing over the object; nor by treating the embryo as a pure object, but rather by questioning it like a living partner, in a dialogue".

Spemann was no chemist, nor yet a physiologist (as the story of the water makes clear), and only towards the end of his career did the search for a chemical embodiment of the organizer begin, with mixed results. Spemann had regarded the inductions he studied as properties of whole tissues, and barely even considered organization at the cellular level. What had impressed him was that an almost complete second embryo could be induced simply by transplanting a tiny sliver of tissue from one embryo into another. This is curiously holistic biology,

The organizer experiment — Spemann summarized the experiment as "The astounding fact that a secondary embryo may be brought about by transplantation". The upper edge of a blastopore from a lightly pigmented newt gastrula was transplanted to the side of a gastrula of a different species of newt with dark pigmentation, giving a gastrula with two blastopores. The graft 'took' and invaginated at its new site. The resulting embryo had two neural plates, two sets of somites, two guts and so on as can be seen in the cross-section shown here. The pigmentation marker showed that the secondary embryo was composed of cells derived from both donor and recipient tissues. Hence the implant was said to 'organize' a secondary embryonic axis running along the flank of the recipient. This property is confined to the upper edge of the blastopore. It is perhaps the most striking known example of embryonic induction. The illustration, which has been relabelled, is taken from Embryonic Development and Induction, by Hans Spemann (Yale University Press, 1938).



yet meticulously and rigorously dissected on its own terms, as Hamburger makes plain.

What is fascinating and puzzling about this period as seen with modern eyes is the apparently complete lack of communication between geneticists and developmental biologists. T.H. Morgan and his colleagues had laid out the basis of modern genetics by the end of the First World War, and Morgan himself was familiar with the main issues in developmental biology through working in marine stations at Naples and Woods Hole. Yet in his 766-page book *Experimental Embryology* (1927), Morgan devoted a scant four pages to the role of chromosomes in development. Too little was known — "Whether all the genes are active all the time, or whether some of them are more active at certain stages of development than are others, are questions of profound interest". Nor was it then certain whether the chromosomes were "concerned with the making of enzymes". Morgan also devoted considerably less space to embryonic induction than he did to

parthenogenesis, but that is another story.

I would be curious to know whether Spemann and his school ever thought about how genes might shape the tissues, or what kind of form they imagined a complete account of embryonic induction would eventually take. Of course, in the mid-1920s there was little enough known about proteins and enzymes, let alone how genes might control their production. Given this ignorance, it is amazing how far the analysis of determination, differentiation and induction could progress, and a revelation to modern molecular eyes to see how much there is still to explain. My only regret about Hamburger's book is that it stops short of the cloning revolution, and sticks closely to amphibian development. It would be marvellous to hear his views on induction, fields and gradients in their modern dress. □

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Condensed matter

W. R. A. Brown

Heterochromatin: Molecular and Structural Aspects. Edited by Ram S. Verma. Cambridge University Press:1988. Pp.301. £30, \$49.50.

AT THE end of a eukaryotic mitosis, most of the chromatin in the cell decondenses. Some does not and remains genetically inactive — this material, with its distinctive cytological properties, is called heterochromatin.

Until recently there has been a gap between the chromosomal features that can be described with the help of a microscope and the size of DNA amenable to molecular analysis. Now, however, pulsed-field gel electrophoresis and molecular cloning in artificial yeast chromosomes are making it possible to analyse megabase pair stretches of DNA, and thus to develop molecular maps of entire eukaryotic chromosomes. These maps will include the structural information necessary for analysis of eukaryotic chromosome behaviour at a molecular level, a prospect which has brought back to prominence an older, sometimes arcane, cytogenetic literature which depicts a eukaryotic chromosome biology of extraordinary diversity. A further consequence has been the resurgence of interest in a variety of dormant cytogenetical questions, such as those surrounding the functional significance of constitutive heterochromatin.

Heterochromatin is a multi-author

volume which sets out to give a contemporary overview of the subject. Appearing as it does at this singular time, it is likely to attract considerable attention. Sadly, in my view, most readers will find it a disappointment.

The first chapter, "The Biology of Heterochromatin" by B. John, occupies nearly half of the book and is an account of the cytogenetics of heterochromatin in animals. Position-effect variegation, segregation distortion, ectopic pairing, heterochromatin elimination and more are reviewed critically. If you need a guide to the older cytogenetic literature, then this is it.

The remaining six contributions can be considered in three groups. A. Lohe and P. Roberts review the sequence organization of satellite DNA in *Drosophila melanogaster* and other *Drosophila* species; this chapter will be of particular value to those working either on other aspects of *Drosophila* biology or on the satellite DNA of other organisms. The next three chapters describe aspects of our knowledge of the ultrastructure of heterochromatin. Here there is duplication and unresolved contradiction, and it would have been better to have had this interesting and apparently controversial area covered in a single authoritative article. The last two contributions describe two rather specialized aspects of human cytogenetics, the first dealing with the use of restriction endonucleases as reagents to help produce banding patterns in human chromosomes, the second with the techniques for detecting human chromosome heteromorphisms.

Altogether this is an uneven book, both in organization and in the quality of the contents. Much is omitted. The discussion of satellite DNA in *Drosophila* is useful, but why is there no general review of tandemly repeated, simple-sequence DNA in other organisms? The molecular basis of mammalian X-chromosome inactivation is covered only briefly. Heterochromatin in plants is mentioned only in passing. Thus the editor has failed in his goal of putting together a comprehensive account of the subject. Nevertheless John's review alone justifies publication of the book, and will ensure that it has a part in the revitalization of the study of heterochromatin. □

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Swooping down the ages — a flying machine, made of natural materials, and based on drawings of Leonardo da Vinci. The machine features in the Leonardo exhibition which opens at the Hayward Gallery, London, today. The book-of-the-exhibition ("an invaluable guide to Leonardo's universe") is also published today by Yale University Press.

Volumes of human learning on machine learning

Max Bramer

Proceedings of the Fifth International Conference on Machine Learning. Edited by John Laird. *Morgan Kaufmann: 1988.* Pp. 467. Pbk \$24.95. In Britain distributed by Afterhurst, 116 Pentonville Road, London NW1 9JB, £19.95.

EWSL 88: Proceedings of the Third European Working Session on Learning. Edited by Derek Sleeman. *Pitman: 1988.* Pp. 263. Pbk £25.

ARTIFICIAL intelligence (AI) is the branch of computer science concerned with developing systems with sophisticated reasoning capabilities — capabilities which, if possessed by human beings, would be described as intelligent. A decade ago AI was seldom heard of outside the research laboratory or the realms of science fiction. The turning point came in 1981 with the setting up in Japan of the ten-year programme to build 'fifth generation' computers for the 1990s, in which "intelligence will be greatly improved to approach that of a human being".

Although it may have seemed a heretical idea at that time, AI systems (for problem solving, computer vision, natural-language processing and other applications) are becoming part of the mainstream of computing. There are now hundreds (maybe thousands) of so-called expert systems for practical use in medicine, geology, chemistry and, increasingly, finance. Essentially, the construction of an expert system involves the elicitation of knowledge from a subject expert and its representation in a computer program, often in the form of rules. Obtaining such knowledge is often a time-consuming and error-prone activity, however, and increasingly attention has turned to finding ways in which this process may be automated. Such considerations provide one — although by no means the only — motivation for the recent resurgence of interest in machine learning.

Human intelligence

Although the exact nature of human intelligence remains unclear (and is likely to do so for a long time), the ability to learn is undoubtedly a crucial element, and a multi-faceted one. To list only some possibilities, human learning can be by rote, by trial and error, by discovery, by generalizing from a series of example cases or by a detailed analysis of a single specific case. Humans can learn by self-instruction or by being taught. Teaching can take the form of imparting an under-

lying theory and/or showing examples of good (or bad) practice.

Much human learning takes place by generalizing from a series of examples. There are many ways in which this can occur: the examples can be presented in a carefully chosen sequence by a teacher, can be chosen at random or in some situations can be self-selecting (for example, patients attending a doctor's surgery). The learner can examine a collection of examples as a whole to develop a strategy for all future cases, or can begin with a simple strategy which is then refined incrementally from further examples as he or she goes along. These and other aspects (such as inductive reasoning) of the human skill known as learning are reflected in the lines of research currently being pursued in machine learning.

Two forms of machine-learning research for which the link with human behaviour is less apparent are 'connectionist' and 'genetic' learning. Connectionist learning is closely related to work on neural nets, a field which has become a bandwagon in the past year or so. Although some interesting results have emerged, it is difficult to evaluate the work concerned without considering the very strong claims which many of its proponents have made about modelling the physiological workings of the human brain. Such claims are notoriously easy to make, but much more difficult to justify. I myself remain unconvinced by them.

The term 'genetic' learning originates from the observation that natural (biological) selection can be regarded as a form of learning, where a simple yet powerful learning strategy is applied repeatedly to a continuous stream of input, whilst the system moves from a rudimentary state (such as a simple single-cell organism) to increasingly more effective ones (such as *Homo sapiens*). In machine learning, genetic algorithms embody some model of natural selection and genetics as their principal learning mechanism. Such algorithms have successfully been applied to tasks like the evaluation of the worth of a position in a game of checkers. Unfortunately, as with connectionist learning, the systems that result are likely to be inscrutable to people, however well they perform their given tasks.

The books under review are the proceedings of two conferences which took place in 1988. That edited by John Laird contains papers presented at the University of Michigan in June; 49 contributions are included, the overall stress being on theory rather than applications.

The way that conference proceedings are broken down into sections is often a good indicator of the balance of research in a particular field at a particular time. In this case, there are 15 papers on empirical learning, seven on explanation-based learning, one on case-based learning, five

on machine discovery, four on connectionist learning, five on genetic learning, three on integrating explanation-based and empirical learning, and nine on other topics. Of particular interest are a number of contributions on the well-known ID3 algorithm for constructing decision trees from large collections of examples, a technique which has been strongly advocated as a powerful way for developing the decision rules to use in expert systems.

Goals and experiments

The EWSL proceedings, which come from a meeting held in Glasgow in October, contain 21 papers. Unfortunately they have not been grouped into sections and have simply been arranged in alphabetical order of author, thus losing whatever structure they had when presented at the conference. As a result two valuable, general articles are buried in the middle of the text when they could usefully have been placed at the beginning as an introduction. These are Donald Michie's "Machine Learning in the Next Five Years", in which he considers the past, present and future of the subject, and the paper by Dennis Kibler and Pat Langley on "Machine Learning as an Experimental Science", in which the authors take a broad look at the goals that underlie the experimental study of learning systems and the types of experiments necessary to achieve them.

Both books will be required reading for those seriously involved in machine-learning research, and will no doubt be frequently cited in the future. Despite its lack of structure, the EWSL volume is the more accessible and perhaps the more valuable collection. But both suffer from the failings of most such volumes — the papers generally lack the broad context necessary to be meaningful to the general reader, or in many cases the researcher in an adjacent part of the field. At a more mundane level, neither book has a subject index, and there are no cumulative lists of references.

These faults may well be unavoidable in books which are put together quickly, so that they can be handed out at a particular conference. But it is surely not asking too much that they should be remedied before the volumes are sold to a wider technical readership. It may seem harsh to criticize the publishers for a sin which is increasingly widespread in the AI world, and doubtless elsewhere. But the lack of some post-conference editing has undoubtedly resulted in two books which are less valuable (if more timely) than they otherwise might have been. □

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Explanation and definition

Alan Isaacs

The Harper Dictionary of Science in Everyday Language. By Herman Schneider and Leo Schneider. Harper & Row: 1988. Pp. 309. \$25.

The American Heritage Dictionary of Science. Edited by Robert K. Barnhart. Houghton Mifflin: 1988. Pp. 740. \$19.95.

THESE two books, both called dictionaries of science, are about as dissimilar as any two books claiming to be dictionaries of anything could be. The essential difference is one of the intended audiences.

The title of the Harper book is plain enough, and the blurb tells us that here is "a book that explains scientific terms and techniques with wit and charm and, most of all, in plain English". Although one may find it hard to imagine anyone being either witty or charming in scientific explanation, the Schneiders have been fairly successful in making fairly difficult concepts fairly lucid to fairly intelligent non-scientists. Interferometry, for example, is quite ingeniously explained, so too are computerized axial tomography and boolean algebra. Indeed, there are some extremely good explanations throughout the book, which one could well imagine being helpful to any layman willing to put up with the wit and charm that go with them.

The authors say (in the preface) that their dictionary came into being because a neighbour was confused by standard definitions of entropy — that the only people capable of understanding these definitions would be scientists. So their own entry begins wittily with T.S. Eliot's "This is the way the world ends..." and launches off charmingly into a piece about "the majestic rotation of galaxies". They do not record how their neighbour reacted; one cannot help wondering if he was much the wiser. At least it is clear from the entry — explanation without definition — at whom it is aimed.

Who, though, will use the American Heritage dictionary? On the inside flap of the jacket it is asserted that the book "bridges the gap between the oversimplified and the highly technical reference", while in his preface the editor notes that it is designed to "support" the student. One hopes he means that the biological entries are designed to help the physical scientist, and vice versa? If that is the case, the book could well serve a useful purpose. But a glance at the entry for entropy is not very reassuring if he doesn't mean this. The definition eschews explanation: "a measure of the unavailability of thermal energy for doing mechanical work", a not

very helpful quotation from L.K. Runnels, and that is all. Surely, the student of physical science would want something more than that. Some attempt at explanation on the lines of $\Delta S = \Delta Q/T$, at the very least.

Another surprising aspect of Barnhart's book is the treatment of chemistry, which is both inconsistent and out of date. For example, there is no entry for ethanal and that for acetaldehyde does not even refer to this systematic name. There is, however, an entry for ethanol, which is given as an "also called" under ethyl alcohol. Ethene has an entry cross-referring it to ethylene, but the entry at ethylene makes no mention of ethene. In view of the international change from trivial to systematic names, it should be one of the

prime tasks for a dictionary at this level to deal correctly with the relationship between the two systems, especially if it is for school and college libraries.

That neither dictionary has solved the problem of either explaining or defining entropy for the layman, the life scientist or the physical scientist, may not be a total condemnation of either. It is a hard concept to get across in any depth without mathematics. Moreover, both books contain plenty of other useful explanations (Harper) and definitions (American Heritage). □

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Cycling essays

Edward Rybicki

Portraits of Viruses: A History of Virology. Edited by F. Fenner and A. Gibbs. Karger: 1988. Pp.344. SwFr. 147, £66.90, \$98.

SINCE I began teaching virology in 1981, I have come across only one book that purported to be a history of the subject — and that is now sadly out of date. It was pleasing, therefore, to hear that Karger were to bring out the volume under review. My enthusiasm dwindled somewhat when I realized that the book consisted of the 15 "Portraits of Viruses" that have been published in *Intervirology* over the years since 1979, and that I had already read most of them. The essays were written by distinguished and long-serving virologists, and are often as much personal scientific biographies (especially those by Heinz Fraenkel-Conrat on tobacco mosaic virus, and Richard Lister on tobacco rattle virus) as they are contributions to the history of research on a particular group of viruses.

In the preface, the editors say that "this collection of essays has some notable gaps". This is very true: there are no reoviruses here, nor any polyoma- or adenoviruses, and there is only one retrovirus. Worse, the portraits have not been updated or expanded; all of them have been reproduced directly from the journal. The editors say that "for contributions written several years ago, we asked the authors to provide a brief addendum...". In fact, only the portraits of the poxviruses (F. Fenner, 1979) and the picornaviruses (J. Melnick, 1983) have such an addendum. The failure to bring the articles up to date is more excusable when their authors have since died — as have Macfarlane Burnet (influenza virus A), Peter Wildy (herpesviruses) and Basil Kassanis (tobacco necrosis virus and its

satellite virus) — but it was remiss of the editors not to have insisted that contributors still living should do so.

The most recent essays were published in *Intervirology* in 1986; seven of the others appeared between 1979 and 1981. Thus it is that William Hayes does not include the exciting developments of the past few years, such as the sequencing of lambda and its development as a powerful tool in the recombinant-DNA armoury; Fraenkel-Conrat neglects the sequencing of the tobacco-mosaic-virus genome; in Burnet's essay there is no mention of the cDNA cloning and sequencing of many influenza virus HA and NA genes, nor of the synthetic peptide and monoclonal antibody work on the unveiling of antigen drift; and T. O. Diener does not tell of the elucidation of the replication and self-cleavage mechanisms of viroids. The essays themselves vary from the very personal and relatively sketchy, to the more formal and comprehensive review-type treatments given to poxviruses, picornaviruses and especially to Rous sarcoma virus (J. Svoboda): these have already proved helpful in my teaching, and remain as valuable references; I shall not be referring much to the other chapters.

These points apart, I have no complaints about the "Portraits of Viruses" series. But I do question whether it was necessary to put together this collection, or whether it will be worthwhile (the editors' hopes notwithstanding) to do so again in the future. The series of portraits always was, and remains, at best fragmentary and anecdotal. Publication of the essays in *Intervirology* is sufficient, in my view, to safeguard their authors' contributions to the body of background material which, one hopes, will be used by others for the writing of fuller accounts of the history of virology. □

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Claims on tissue plasminogen activator

R. Stephen Crespi

A 'master' patent on a recombinant protein product has twice been overturned in British courts. The consequences may be far-reaching.

THE struggle between Genentech and the Wellcome Foundation over Genentech's British patent for tissue plasminogen activator is of keen interest world-wide. Tissue plasminogen activator (TPA) converts plasminogen into plasmin, the enzyme that breaks down fibrin clots formed in coronary thrombosis, and has a large potential market. Britain is the arena for the first patent litigation on this biotechnology product, but the issues at stake transcend national boundaries and the judgement of the British courts will have international influence.

The patent in suit was obtained through the British system, but Genentech also has a parallel patent application in the European Patent Office, which will probably be granted no later than mid-1989. Depending on the claims that issue, the European patent will give Genentech an apparent second chance to protect its British market if the British national patent, which was rejected in July 1987 in the British Patents Court and again by the Court of Appeal in October 1988, is finally held invalid on appeal to the House of Lords. At the time of writing Genentech has yet to appeal.

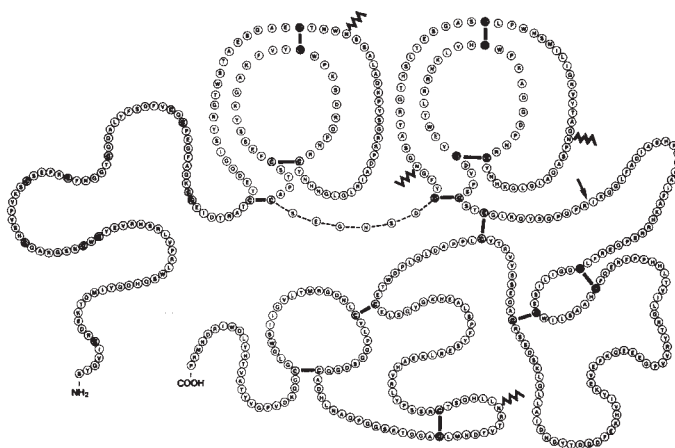
Genentech has a corresponding patent in the United States, which was granted in August 1988. This claims the DNA sequence that encodes TPA as well as expression vectors and transformed microorganisms containing this gene, but not the final TPA product. Litigation on the US patent has also begun.

The significance of the TPA case in Britain is that Genentech is strenuously pursuing patent claims for the recombinant protein product as such, and for correspondingly broad processes, rather than a claim for any detailed method limited to particular plasmids chosen to carry the TPA cDNA into the particular host cells used by Genentech to express the protein product. The case is therefore a prototype for other patents in the pipeline covering other blood proteins — including factor VIII, antithrombin and serum albumin — and may well be a pointer to the evaluation of the patent system in relation to similar biotechnology products. It is not surprising, in view of the decisions handed down so far, that some commentators have suggested devising another type of legal protection that would recognize with more certainty the achievements of those who are first to succeed in producing valu-

able proteins by genetic engineering.

To appreciate the issues in the TPA case, it is necessary to understand how patents are constructed. A patent specification consists of two parts: first, the technical description, which sets out the nature of the invention and instructs the skilled worker how to reproduce it; second, the legal definitions, known as the 'claims', which circumscribe the protection given by the patent. The claims are verbal formulae defining the protected

Patent disputes centre almost entirely upon the claims. The patentability conditions of novelty, inventiveness and industrial application or utility are applied solely to the claimed subject matter. The other main condition is that the inventor must provide a sufficient or 'enabling' disclosure. The disclosure requirement applies to the technical description; and if a claim is so broad as to cover subject matter for which the directions given to make the product or perform the process



Desirable form — diagram of the potential structure of human TPA, published by Pennica et al. after the priority date of Genentech's first patent application (Nature 301, 219; 1983).

technology in terms of process, product, use or some other category. Here the wording is important, although the courts have always allowed some elasticity of interpretation in order to catch clever evaders of the literal text. The European Patent Convention tries to strike a balance between the extremes of tight literal wording, on the one hand, and woolly statements of uncertain scope on the other.

A claim to the product itself (a *per se* claim) is possible when the product is new (that is, it has not been previously described). But where the product is not new *per se*, but only the process for making it is new, it is possible to claim the product as made by the particular process (a product-by-process claim). As human TPA was not a totally new substance at the priority date of Genentech's first patent application (in the United States), it was appropriate to use the more restricted product claim, as exemplified by claim 3 in the TPA patent, which reads: "Human tissue plasminogen activator as produced by recombinant DNA technology".

are seriously defective, the patent will be invalid.

Here, I should dispose of a common misunderstanding. It is incorrect to assume that products of nature cannot be patented on the grounds that, by reason of their previous existence in nature, they cannot be novel. Patent law has become much more liberal on this point by taking into account the merit of first discovery of a natural substance, its isolation and provision of a product in a form useful for therapeutic or other purposes. The guidelines of the European Patent Office follow this approach and endorse the patentability of natural products in the proper circumstances. Of course, TPA had been isolated from human tissue before Genentech began work, and the identical product had also been produced by culture of the Bowes melanoma cell line. The TPA claim quoted above was therefore appropriate. As defined, the product is not one of nature but of a non-natural process.

Genentech based its case on the position stated in the patent itself:

The present invention arises in part from the discovery of the DNA sequence and deduced amino acid sequence of human plasminogen activator. This discovery enabled the production of human plasminogen activator via the application of recombinant DNA technology, in turn, enabling the production of sufficient quality and quantity of material to initiate and conduct animal and clinical testing as prerequisites to market approval, unimpeded by the restrictions necessarily inherent in the isolation methods hitherto employed involving production and extraction from existing cell culture. This invention is directed to these associated embodiments in all respects.

The Patents Court held that broad product claims such as claim 3 would only have been acceptable if the patentee had been the first to discover TPA or its desirable properties. Although Genentech had been the first to identify the DNA-coding sequence and to deduce the amino-acid sequence of the protein (in the court said to be "a remarkable job of work" which "involved laborious and costly effort") it had already been recognized by other workers as desirable to produce TPA in quantity by recombinant DNA methods. Genentech's product claims were therefore too wide in being directed to a possible and obviously desirable end that could be reached by routes on which only limited guidance had been given. Only a limited process claim could be upheld in these circumstances.

Last October, at least four crucial questions emerged in the Court of Appeal. What precisely did Genentech *invent* (rather than merely *discover*)? What scope of claim would properly reflect the invention? Was the claim to recombinant TPA obvious to the skilled worker at the relevant date? And what level of skill and knowledge would the hypothetical ordinary skilled worker be assumed to have in relation to the previous question?

Key contribution?

Genentech stuck to the assertion that the discovery of the sequence was the key contribution, the 'one log holding up the log jam' in the way of progress to the desired product. But both British and European law state that a discovery, as such, is not patentable, and it is universal law that you cannot patent knowledge itself but only its practical application. One judge floated the idea of subtracting the discovery element and seeing whether what was left amounted to an inventive step. This is a dangerous line to follow for any invention in the natural sciences, and fortunately the final judgement will not rest on this type of analysis.

On scope of claim, Genentech pursued a very bold line. The company argued that the discovery of the cDNA sequence led not only to the production of the recombinant form of natural TPA having the amino-acid sequence 1 to 527 shown in Fig. 5 of the patent drawings, but also

opened the way to modifications of the natural molecule (for example, by site-directed mutagenesis of the underlying DNA) which preserved the essential TPA-type activity. This extension of the claim was of no great importance as regards Wellcome's intended product, but shows that Genentech was seeking to dominate a wide range of second- and subsequent-generation products in this field. To claim for the TPA patent the status of a master patent on the basis of discovering the sequence of TPA alone was remarkably daring. The Court of Appeal found this far-reaching proposal unacceptable and was not prepared to sustain claim 3 even if limited to the recombinant equivalent of the natural product. Thus, one judge pronounced: the "contention that Genentech should be protected against any use of this information howsoever this may be achieved in the future is . . . not a claim to a method embracing the discovery but rather a claim for protection of the discovery as such".

In patent disputes over new chemical compounds, it has been argued that something is obvious if a number of research groups are working along similar lines. This argument has not been well received. In the TPA case, however, it succeeded, as seen in the following extracts from the judgement:

It is agreed between the experts that at the relevant time, in early May 1982, the production of quantities of TPA adequate to treat human patients suffering from blood clots was known to be a desirable objective and it is further agreed that the Bowes melanoma cell-line was then available and was known to be a source of TPA and of the corresponding mRNA. It was of course then known that the nucleotide and amino-acid sequences of TPA existed, though their composition was not known. All the steps taken by Genentech in finding out the composition of the sequences and applying that knowledge to produce human TPA, as defined in the specification, by recombinant DNA technology were applications of known technology, and no step was by itself inventive. . . .

. . . it was indeed obvious, in my judgement, to the person skilled in the art to set out to produce human TPA by recombinant DNA technology. At least four teams did just that at about the same time. The evidence of those at Leuven and Umea [other research groups] as to their choice of projects is particularly relevant. The end was a known desideratum, and to proceed by oligonucleotide probing was, if not the first choice of each team, an early choice which lay in the way, ready to hand.

Wellcome thus persuaded two of the three judges in the Court of Appeal that the product claims were invalid not only because they were conceptually obvious but also because their practical realization involved no inventive step. Wellcome did not fare so well with the additional contention that the claims were also of unfairly wide scope in light of the technical description. This is a matter that the Patent

Office can raise when it examines a patent application. But once a patent has been granted, the Court of Appeal held that it cannot be re-opened under the permitted grounds on which patents can be revoked. This conclusion stands in sharp contrast to the long-standing tradition in British patent law that the courts can also consider the question of fairness of the scope of a claim in the light of the patent description. If Wellcome counter-appeals against this point, the House of Lords must decide whether the 1977 Patents Act denies to the courts a remedy open to the lowest level of authority in the British patent system.

New problem

What then is unique or new about the TPA case? One Appeal judge said:

. . . we are here dealing with a new problem. New not only because the technology is very recent, but also because the problems arising from the manufacture of an existing substance with known properties, by the application of a known technology, through the medium of a new discovery and novel intermediate artifacts (the vectors) has never (so far as I can see) been considered by the courts.

The final answer, however, has been clear and unanimous from all judges in the case, even though their individual approaches vary distinctly. The same judge also said:

. . . the importance of the present action far outstrips the ultimate decision itself, for it is impossible to arrive at a decision without expressing conclusions which apply to this fledgling industry as a whole, and which (if reflected in courts elsewhere in the European Community and outside) may have an important bearing on its future.

However hard the judgements may seem, they simply result from the application of standard principles of patent law to the cloning of DNA that encodes naturally occurring proteins. For some time it has been an open question whether such cloning involves invention as distinct from experimental skill and the application of sufficient resources. But it is difficult to see how a different legal system would cope better with the situation. Copyright as an alternative to patent law is a blind alley in relation to DNA sequences, and no other new system is yet on any lawyer's drawing board. Moreover, the difficulties are not nearly as great for newly isolated natural proteins and the products of protein engineering; here the question of inventiveness will return to the more familiar field of new compound chemistry. Given that the complexities of the Genentech case stem from transitional problems, it is premature to despair of the patent system for the protection of innovation in biotechnology. □

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The physics of dense nuclear matter from supernovae to quark gluon plasma

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At high density, nuclear matter is better regarded as a dense fluid rather than a collection of individual protons and neutrons. A good understanding of the properties of nuclear matter, important in the physics of neutron stars and the Big Bang as well as heavy atomic nuclei, is now emerging.

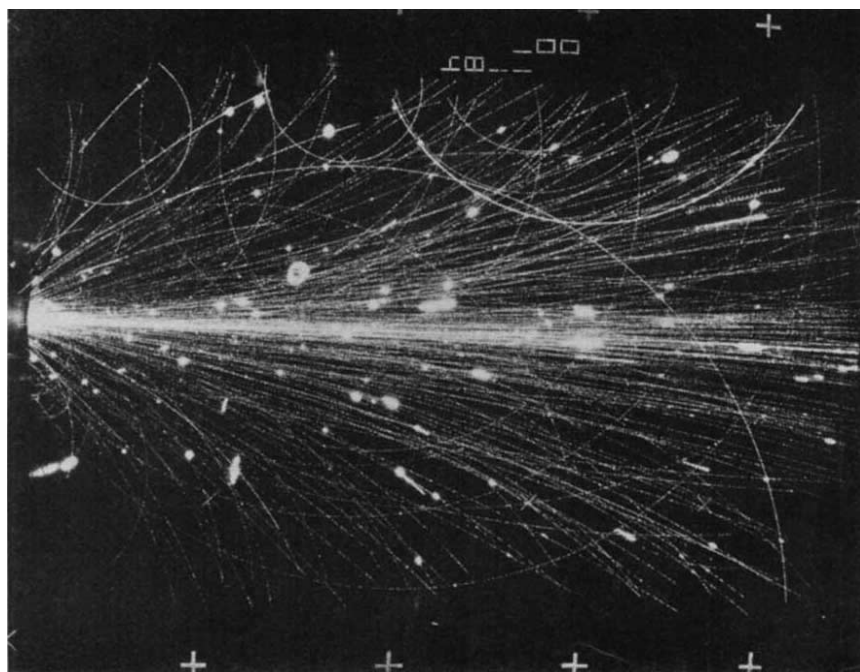
IN light atomic nuclei, the constituent protons and neutrons retain some of their individual identity and occupy distinct energy states, but in the interior of heavy nuclei, where the density is higher, the available energy states are closer together and more numerous, and the neutrons and protons form a continuous, fluid-like medium known as nuclear matter. Ground-state nuclear matter is stable because the powerful attractive and repulsive components of the strong interaction nearly cancel out. The additional electromagnetic interaction—Coulomb repulsion between the charged protons—causes a stability limit at nucleon number $A \approx 210$, close to that of lead and bismuth. Were it not for this interaction, nuclear matter could form stable volumes of any microscopic or macroscopic size. Stability returns only when the atomic number becomes very large, $A \geq 10^{57}$. Then gravitational attraction is enough to balance the repulsion of the strong interaction, permitting the formation of neutron stars.

Despite decades of research in nuclear physics, we still do not understand nuclear matter very well. Until recently, the physicists' task was seen largely as the construction of detailed models for isolated nuclear species and their excited states, and for the interactions between them. Nuclear matter was regarded as an abstract extrapolation from studies of nuclear structure.

This attitude changed about 20 years ago, when astrophysicists began to develop semi-quantitative models for supernova dynamics, neutron stars and the evolution of the Big Bang, all of which demanded detailed understanding of the continuum properties of extended nuclear matter or, more generally, of strongly interacting matter at high density. This need prompted the research described here.

The ground-state density in nuclei is $2.7 \times 10^{14} \text{ g cm}^{-3}$, corresponding to a numerical nucleon density of $\rho_0 = 0.15 \text{ fm}^{-3}$ ($1 \text{ fm} = 10^{-15} \text{ m}$) and an energy density $\epsilon_0 = 0.14 \text{ GeV fm}^{-3}$. Familiar nuclear reactions such as fission, fusion and alpha-decay proceed with only small changes in density, of a few per cent: nuclear matter flows gently between its various 'droplet' species. Type II supernovae (that is, those occurring among young, massive (Population I) stars) may, on the other hand, reach densities of about $4\rho_0$, and still higher densities are proposed for the interior of heavy neutron stars. To understand supernova dynamics, the stability and static architecture of neutron stars, and the critical limits governing black-hole formation, we need to know the macroscopic properties of nuclear matter, such as compressibility, specific heat and entropy density. In short, we need to know the equation of state of nuclear matter (the relationship between pressure, density and

A streamer-chamber photograph of a central collision between ^{16}O and Pb at the CERN SPS, with an energy of 200 GeV per nucleon. It illustrates the high multiplicity of final-state events which must be scrutinized for evidence of the transient formation of quark matter during the collision. The magnetic field in the detector separates positive and negative particles, bending them according to their momenta, and track brilliance measurements distinguish particle types. The tracks in the pencil surrounding the beam axis, corresponding to the forward part of the phase space, cannot be resolved, but are of minor significance in the analysis.



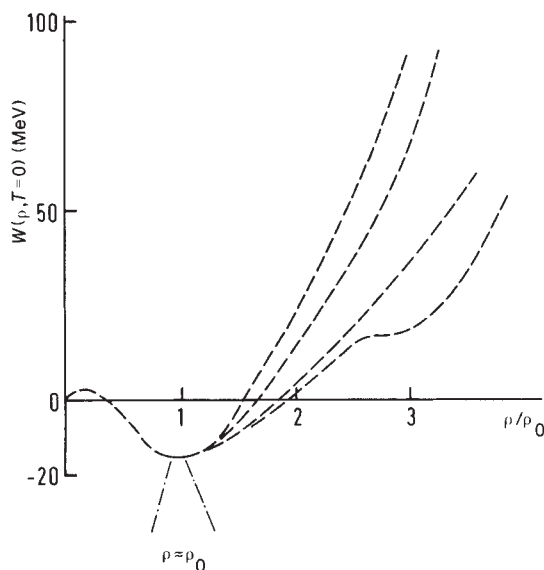


Fig. 1 Sketch of the energy-density function (the energy per particle) of nuclear matter. The internal energy per nucleon of cold ($T=0$) matter has a well-known minimum at density $\rho = \rho_0$, corresponding to the matter inside stable nuclei. Towards higher densities the energy should increase.

temperature) over a wide range of density, far beyond that of the nuclear interior.

History

The work described in this article had the goal of achieving high compression of nuclear matter in the laboratory by colliding heavy nuclear projectiles 'head-on' at relativistic energy. The concept of shock compression in such heavy-ion collisions was analysed theoretically between 1973 and 1975 by H.A. Bethe at Cornell, G. Chapline at Lawrence Livermore Laboratory, W. Greiner at Frankfurt and their collaborators¹⁻³. At the same time, experimental investigations were made possible by construction of the Bevalac accelerator at Lawrence Berkeley Laboratory (LBL). By combining a low-energy heavy-ion linear accelerator with the old high-energy proton synchrotron (Bevatron) the Bevalac first provided beams of nuclei up to $A = 40$ (calcium) at energies up to 2 GeV per projectile nucleon. With a total kinetic energy about twice the rest-mass of the nucleus, the projectiles move at relativistic velocity ($v \approx 0.9c$). Technical improvements circumvented the limitation to $A \leq 40$, permitting beams of gold and uranium nuclei to be produced. Similar work at Dubna, Soviet Union, reached higher energy (3.5 GeV per nucleon) but only for nuclei up to $A = 20$ (neon), adding an element of competition to the emerging field of relativistic heavy-ion physics.

At Berkeley, the search for the nuclear equation of state was carried out, in particular, by three major international collaborations of physicists with backgrounds in both nuclear and particle physics. A collaboration between Japan and the United States focused on the development of magnetic spectrometers, and two German-US groups constructed complementary types of 4π -detector systems, the visual-track-recording streamer chamber⁴ and the electronic Plastic Ball detector⁵, which simultaneously measure all particles created in a collision event. By the early 1980s, the German-US collaboration had succeeded in an initial demonstration of the shock-compression mechanism and arrived at a first semi-empirical estimate of the equation of state in the density domain $1.5 < \rho/\rho_0 < 3.5$ (refs 4, 5). But a different conclusion was reached at about the same time from the dynamical model for the so-called prompt supernova-bounce mechanism⁶, which indicated an ultra-soft (very low compression energy) equation of state. This was in strong disagreement

with the very stiff (high compression energy) equation of state indicated by the results from heavy-ion collisions. Subsequent theoretical work has arrived at a possible reconciliation of the two results. A new generation of refined experimental investigations, both at LBL and with the synchrotron-storage ring combination which is now under construction at GSI Darmstadt, will be based on these ideas.

Experiments in the GeV-per-nucleon energy domain move far away from familiar ground-state nuclear matter but do not reach the very limit of dense matter consisting of hadrons (nucleons, baryon resonances, pions, kaons and so on). The gauge theory of the strong interaction, quantum chromodynamics (QCD), leads one to expect a phase transition at a high hadronic packing density, in which the quarks and gluons escape from their confinement within hadron 'bags' and move instead through an extended quark-gluon medium termed quark matter⁷. A modern statistical method used to obtain the QCD equation of state, lattice-gauge QCD, has predicted that this phase transition from hadronic to quark matter should occur at energy densities of $\varepsilon \approx 2-3 \text{ GeV fm}^{-3}$, about 20 times the ground-state nuclear-matter energy density⁸. The Bevalac and future similar accelerators do not reach beyond $\sim 0.7 \text{ GeV fm}^{-3}$.

The quark-gluon plasma is a primordial medium whose properties are dictated by the strong force between quarks. It probably existed during the first microsecond (the quark era) of the Big Bang, just before the 'freezeout' into recognizable hadrons. Demonstration of its existence and determination of its critical density, temperature and transition enthalpy would not only provide a novel check for QCD but would also aid our understanding of the composition and distribution of matter in the hadronic period of the Universe, which still exists today. Far higher accelerator energies are required to probe this state. A programme of heavy-ion reaction studies was thus begun at the Alternating Gradient Synchrotron (AGS) at Brookhaven National Laboratory, and at the Super Proton Synchrotron (SPS) at CERN, Geneva. Both have beams of $A \approx 30$ (^{28}Si , ^{32}S), the AGS reaching 14.5 GeV per nucleon, the SPS up to 200 GeV per nucleon. In first runs during 1986-87, new international experimental collaborations have employed vastly more complicated and diverse instruments than earlier studies at the Bevalac and in Dubna. The first results seem to indicate that energy densities of $2-3 \text{ GeV fm}^{-3}$ are indeed attained and some initial observations are briefly described at the end of this article.

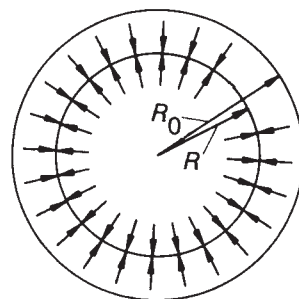
The nuclear equation of state

The mechanics of continuous media are described in the equation of state, which specifies the pressure as a function of density and temperature, $P(\rho, T)$. For microscopic models, the energy per particle, $W(\rho, T)$ is a more convenient quantity. Knowledge of W is, in principle, equivalent to knowing the equation of state, because

$$P(\rho, T) = \rho^2 \frac{\partial W(\rho, T)}{\partial \rho} \quad (\text{at constant entropy}) \quad (1)$$

and the term equation of state is often loosely employed for the function $W(\rho, T)$. In the absence of thermal excitation, that is, for $T=0$ states such as nuclear ground states or neutron-star

Fig. 2 Sketch of hydrostatic equilibrium in a neutron star of radius R_0 . At each subshell with radius R the compressional nuclear matter pressure has to counterbalance the gravitational inward pressure of the layer from R to R_0 .



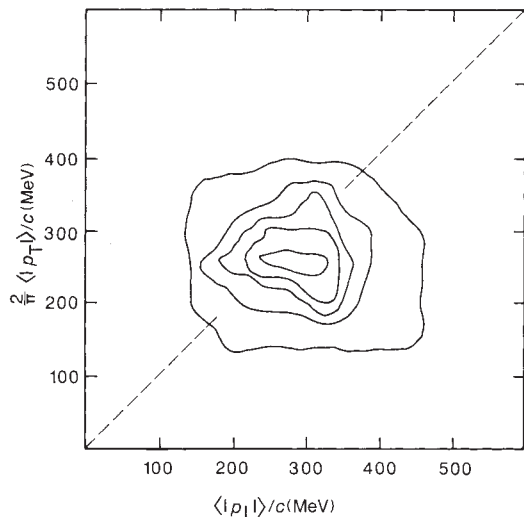


Fig. 3 The achievement of a temporary thermal equilibrium in nucleus-nucleus collisions requires that the momentum of the resultant body in the mean transverse ($\langle p_T \rangle$) and longitudinal ($\langle p_L \rangle$) directions are equal. The data for collisions of ^{40}Ar and Pb at 0.8 GeV per nucleon demonstrate that this is fulfilled on average: the contour lines of event frequency (each spaced by a factor of two) show a 'mountain' at the diagonal.

matter, it is sufficient to know the compressional energy $W(\rho, T=0)$, from which the $T=0$ equation of state follows:

$$P(\rho, T=0) = \rho^2 \frac{dW(\rho, T=0)}{d\rho} \quad (2)$$

Figure 1 illustrates the qualitative expectations for $W(\rho, T=0)$ of nuclear matter. The minimum at $\rho = \rho_0$ represents the stability of ground-state matter: $P(\rho_0, T=0)=0$ from equation (2). $W(\rho_0, T=0)$ is the familiar mean binding energy per nucleon, W_0 , given by the Bethe-Weizsäcker formula. The assumption of a linear response to perturbations about $\rho = \rho_0$ yields

$$W(\rho, T=0) = \frac{K}{18\rho_0^2} (\rho - \rho_0)^2 + W_0 \quad (3)$$

$P(\rho)$ then crosses the $P=0$ axis with slope $(dP/d\rho)_{\rho=0} = K/9$, and the stiffness of the medium is given by the compressibility K , which also fixes the sound velocity. An estimate of K can be obtained from the excitation energy of the nuclear monopole vibrational state, which has been successfully described as a collective density oscillation about ρ_0 . The canonical semi-empirical value thus derived was previously considered to be $K = 220 \pm 30$ MeV (ref. 9) but new data and theory now indicate $K = 300 \pm 25$ MeV (ref. 10), a compression response which must be considered 'stiff'; a 'soft' response corresponds to $K \approx 150$ MeV.

So what is there left to do? Figure 1 shows that there are vastly different possible forms for W at $\rho > 1.5\rho_0$. The monopole vibration covers only a tiny density interval, $\rho = \rho_0 \pm 2\%$, in which it confirms the parabolic shape (equation 3) and yields a value for the constant K . W cannot remain parabolic at high ρ/ρ_0 because nuclear matter would behave acausally (sound speed $v_s > c$) even at neutron-star densities. The parameter K alone does not describe $W(\rho, T=0)$ and may be totally irrelevant for $\rho > 2\rho_0$. In this domain W should of course grow with ρ because the short-range nuclear interaction is repulsive. This can be understood qualitatively by observing that the average distance r of neighbouring nucleons is about 1.8 fm in ground-state, $\rho = \rho_0$ matter, which is just twice the nucleon 'radius' $R = 0.9$ fm found from electron scattering¹¹. At $\rho = \rho_0$, the nucleons are thus already tightly packed, and at fourfold compression they would get squeezed to $r \approx 1.1$ fm, so that their internal quark wavefunctions would overlap significantly. The

Pauli principle would then force some of the quarks up to orbits of higher energy and this, crudely speaking, is the energy expended during compression. W thus grows with ρ —but how steeply?

Consider neutron stars, for example. Figure 2 illustrates the relevance of the equation of state for radial density distribution and stability. Hydrostatic equilibrium demands that the inward gravitational pressure is compensated by an equally large outward pressure from nuclear-matter incompressibility, at each radial shell of thickness $R_0 - R$ (where R_0 is the radius of the star). $P(\rho, T=0)$ thus determines the stellar density distribution $\rho(R)$. If the total mass is too high or the equation of state is too soft at high ρ , there is no stable solution and gravitation creates a black hole. If the strong interaction could be removed, the Fermi energy of the degenerate neutron gas alone would let $W(\rho, T=0)$ increase as $\rho^{2/3}$, and the maximum mass would be the well-known Chandrasekhar limit of 1.4 solar masses ($M_\odot$) (refs 12, 13). But neutron stars of up to $2 M_\odot$ are known¹⁴, so the strong interaction must add to the repulsion. For $\rho \geq 2\rho_0$, $W(\rho)$ thus grows faster than $\rho^{2/3}$ but slower than ρ^2 .

Shock compression in relativistic collisions

Shock compression occurs when portions of a medium move through each other at supersonic velocities. If two equal-mass heavy nuclei collide head on at a beam energy of 1 GeV per nucleon, the interpenetration velocity of the target and projectile nuclear matter in the centre-of-mass frame is about $0.7c$, well above the velocity of sound or of any other mechanism that could transport energy or matter out of the reaction volume. Matter and energy therefore pile up in the shock zone, producing the desired compression in a 'fireball' volume. The hydrodynamical model of shock formation¹⁻³ predicts a fireball density of $\rho \approx 3\rho_0$ at this energy, a temperature¹⁵ of about 80 MeV ($\sim 10^{12}$ K) and a lifetime of $\sim 5 \times 10^{-23}$ s for heavy colliding nuclei ($A \approx 200$). Afterwards, the compressed zone expands, again passing through $\rho = \rho_0$ and exploding into non-interacting hadrons at $\rho < 0.3\rho_0$. The overall dynamical cycle is a sequence of shock compression, fireball, expansion and freezing out.

It took some time before suitable experimental probes for the high-density phase were devised. Before describing these, it is worth considering why the thermodynamic model should be applicable to these reactions. In particular, this model assumes that the shock volume (the fireball) is at rest in the centre-of-mass frame. The two interpenetrating volumes of nuclear matter thus have to stop down their initial relative motion, producing a fireball in which the transverse and longitudinal motions are equally distributed. This nuclear stopping process arises from elastic and, more effectively, inelastic scattering of the incoming nucleons, which populate the transverse degrees of freedom in the fireball, as well as pion production, isobar excitation and compressional (potential) energy. Essentially all the terms in the energy-density function $W(\rho, T)$ are separate agents creating internal centre-of-mass energy out of initial longitudinal energy. The degree of stopping is easy to check. Figure 3 shows streamer-chamber data¹⁶ for head-on collisions between ^{40}Ar and Pb at 0.8 GeV per nucleon. For each collision, the mean transverse ($\langle p_T \rangle$) and longitudinal ($\langle p_L \rangle$) momentum of all final-state particles was determined, giving one point in the $\langle p_T \rangle$ - $\langle p_L \rangle$ plane. Equipartition requires $\langle p_T \rangle = \pi/2 \langle p_L \rangle$ in the centre-of-mass frame, and the data show that this is fulfilled on average. From the same experiment the total transverse energy is found to be in good agreement with the kinematic prediction under the assumption of complete stopping.

One of the key questions in extracting the W function from the data is that of how the internal energy is shared between thermal motion, compressional energy, newly created rest mass and so on. If we combine the rest-mass (m_0) component with the thermal energy (because the new $m_0 c^2 \rightarrow 0$ as $T \rightarrow 0$), we could expect to determine the compressional energy

$W^{\text{comp}}(\rho, T=0)$ from the known total centre-of-mass (c.m.) energy (assuming full stopping) by an appropriate measurement of the thermal energy fraction W^{therm} :

$$E \text{ per nucleon (lab)} \rightarrow E \text{ per nucleon (c.m.)} \\ \approx W^{\text{comp}}(\rho, T=0) + W^{\text{therm}}(\rho, T). \quad (4)$$

In other words, the ground-state function $W(\rho, \tau=0)$ can be approximately found if the thermal component can be measured and subtracted. The idea that the pion yield closely reflects the thermal energy fraction (intuitively, pions are created out of collision energy, with the potential energy remaining passive)^{2,17} was exploited by the GSI-LBL streamer chamber collaboration^{18,19}. Figure 4 illustrates how the supposed compressional energy was determined from systematic measurement²⁰ of the pion yield in central collisions of equal-mass pairs at $A \approx 40$ (Ar and KCl) and 140 (La and La). The ratio of pions produced to nucleons participating, $\langle n_\pi \rangle / A$, increases linearly with the centre-of-mass energy per nucleon above ~ 100 MeV per nucleon.

A calculation employing a thermochemical pion-production model that contains no potential energy gives a pion yield that increases more steeply with energy than does the experimental data. An arbitrary pion concentration is thus reached at an energy ε' , according to the thermal model, which is lower than the energy ε when that concentration is actually observed. The difference between ε and ε' was interpreted as the potential energy expended in reaching the density $\rho(\varepsilon)$ from initial ground-state density ρ_0 . This simple-minded assumption gives $\varepsilon - \varepsilon' \approx W^{\text{comp}}$ as a function of the centre-of-mass energy ε . From the Hugoniot-Rankine equation of hydrodynamic shock compression¹⁻³, ρ was determined as a function of ε , finally

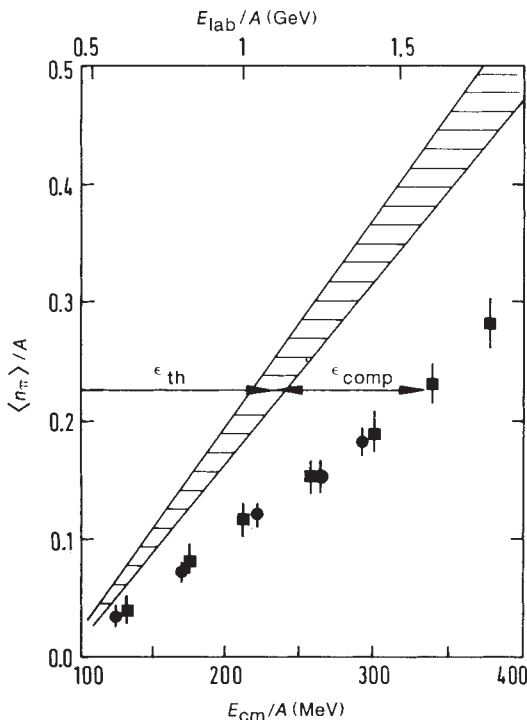


Fig. 4 Pion production rates from the fireball produced in a nuclear collision should be proportional to the thermal energy ε_{th} regardless of the potential energy. Data for the average number of pions produced $\langle n_\pi \rangle$ per nucleon participating in central collisions of Ar and KCl (squares), and La and La, (circles), as a function of the energy per nucleon in the centre-of-mass (E_{cm} , bottom scale) and laboratory (E_{lab} , top scale) frame, are used to estimate the compressional (potential) energy $\varepsilon_{\text{comp}}$ according to equation (4). The hatched region indicates the predictions of thermal models. In these experiments, solid KCl was used as a target as the nearest non-gaseous, non-conducting equivalent to the Ar-atom projectiles (of mass 38).

yielding a semi-empirical estimate of $W(\rho, T=0)$. The results²⁰ and a critique of this procedure are discussed below.

Instead of trying to determine the energy function $W(\rho, T=0)$ why not aim directly at the pressure $P(\rho)$? The long-standing prediction²¹ that collective pressure would lead to a sideways flow of emitted baryons was finally verified by the Plastic Ball collaboration²². Just as a water droplet splashes sideways on impact, nuclear matter develops a pressure field during interpenetration that displaces matter perpendicular to the beam axis. The final-state momentum distribution of all participating nucleons should thus be cigar-shaped in momentum space, a triaxial ellipsoid with its longest axis in the direction of favoured baryon flow. Its angle with respect to the beam axis is called the flow angle. When other variables are kept constant, the flow angle increases with the pressure in the reaction zone and so with the stiffness of the equation of state.

Figure 5 shows the results²³ from the Plastic Ball experiment for the flow angle in collisions between two gold atoms at various energies. This spectrometer has 971 single detectors arranged in a tightly-packed ball to measure particle emission angle, energy and identity for all charged final-state products, and has a planar surface to pick up the small forward angles⁵. The recorded events were first grouped according to their charged-particle or proton multiplicity (number of charged particles or protons created), where a low multiplicity is known to correspond to a peripheral collision and a high one to a central collision. The momentum tensor was then diagonalized for each event, yielding the flow angle Θ . The histograms in figure 5 show the distribution of $\cos \Theta$ for various bins of multiplicity and at five incident energies. In low-multiplicity events, the distribution peaks at $\Theta = 0^\circ$: no preferential sideways flux occurs in grazing collisions, where only the dilute nuclear surfaces interact and there is little compression. A clear sideways peak developed for high-multiplicity events (near-central collisions).

Information about the equation of state can be obtained by comparing the data to theoretical calculations of the reaction dynamics that use a parameterized equation of state. This use of macroscopic models, such as hydrodynamics, and microscopic models that follow the nucleon collision cascades makes it clear that the equation of state is not directly measurable. All results from heavy-ion collisions require auxiliary models and are thus semi-empirical.

Nuclear collisions and astrophysics

The outcome of heavy-ion collision work on the nuclear-matter energy-density function $W(\rho, T=0)$ as it stood in 1986 is shown in Fig. 6, which includes results from the streamer chamber investigation of the 'pion thermometer'¹⁸⁻²⁰ as well as transverse energy data²⁴ measured by the Plastic Ball collaboration using a variation of the assumptions embodied in equation (4). These points agree closely with the W function deduced from fitting the flow angle and related data. The microscopic models employed here²⁵⁻²⁷ were developed mostly by Stöcker, Aichelin and Bertsch as an extension of simple models of collision cascades by including the Pauli principle, microscopic effective nucleon-nucleon interactions and an average mean field, giving the nucleons a potential energy. This type of theory is known as the Vlasov-Uehling-Uhlenbeck (VUU) model. Figure 6 indicates a stiff equation of state. Note, however, that the data points start at a density $\rho/\rho_0 = 1.7$ and give no new insight into the result obtained from the monopole vibration in the immediate vicinity of $\rho = \rho_0$. But, as expected, W is approximately linear in ρ/ρ_0 .

These results can be compared with astrophysical estimates of the equation of state for heavy neutron stars²⁸, also shown in Fig. 6. Analysis of 4U0900-40, a star with mass $\sim 1.85 M_\odot$, gives a much shallower W function. An even softer equation of state has been used by Baron, Cooperstein and Kahana (BCK) in the prompt supernova-bounce model²⁹. The BCK model posits that a type II supernova arises from a compression-shock

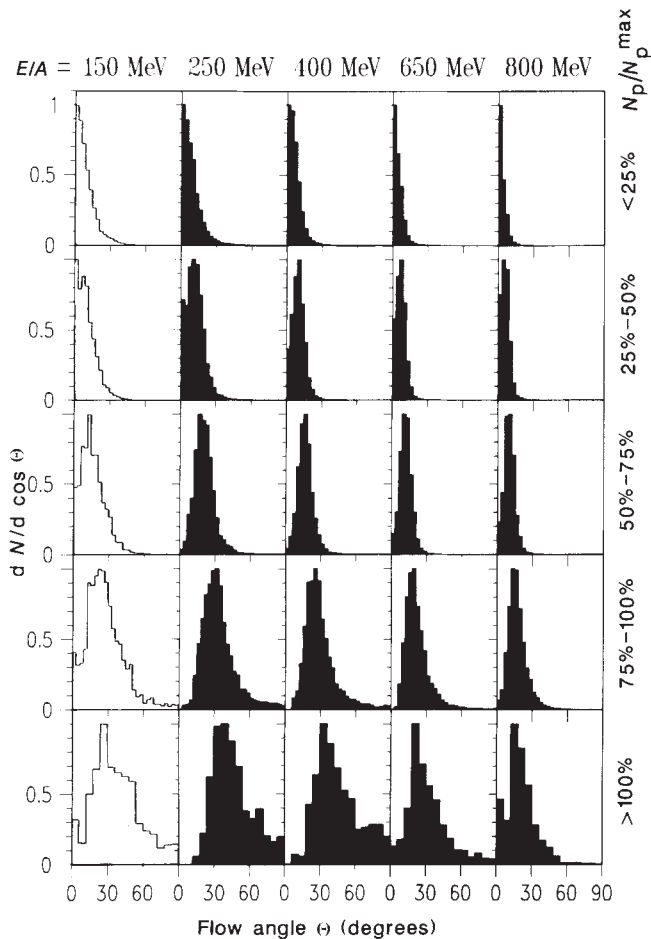


Fig. 5 In nuclear collisions at higher energies, the resultant blob of hot material is increasingly non-spherical. As a measure of this effect, the flow angle Θ between the beam axis and the longest axis of the fireball is measured. The figure plots the distribution of Θ in collisions of two gold atoms at five incident energies. The data are subdivided by multiplicity, the number of protons N_p in each event; five multiplicity bins are shown, with N_p divided by $N_p^{\max}$, the average proton multiplicity in head-on events. N_p can occasionally exceed $N_p^{\max}$. The flow angle peaks at 0° for low N_p (peripheral collisions) but develops a sideways shift at high N_p (central collisions).

explosion cycle of the iron core of a burned-out star of about $12\text{--}15 M_\odot$, where the core has a mass of $\sim 1.4 M_\odot$. The shock explosion has to carry enough energy and matter outward to avoid being stalled by the mass of outer-shell material still falling inward. To create such a massive shock wave the collapse has to continue to very high density (small core radius) and thus traps a large amount of gravitational energy in the shock front that develops at the bounce point. The outer layer of the core then rebounds, the inner sections settling into a residual neutron star. A soft equation of state is required to reach high bounce-point densities, of $\rho \approx 4\rho_0$. The range of W functions that lead to a successful prompt-bounce simulation²⁹ is indicated in Fig. 6.

The first semi-empirical estimates of the energy-density function seem to be as contradictory as the qualitative *a priori* sketches (Fig. 2), but critical consideration of the theoretical models used in the derivation may remove some of the apparent contradictions.

● Nuclear collisions create hot hadronic matter, at temperatures up to 100 MeV, and contain up to 25% of their energy in pions and nucleon resonances. Supernovae, by contrast, are cold, proceeding at temperatures of about 10–15 MeV, neutron stars are at $T=0$, and both are more nearly pure nucleon matter. The fireball nucleons move with a velocity of about $0.5c$ and the nucleon-nucleon potential is strongly velocity-dependent^{30,31}, so nuclear collisions do not reflect at all the

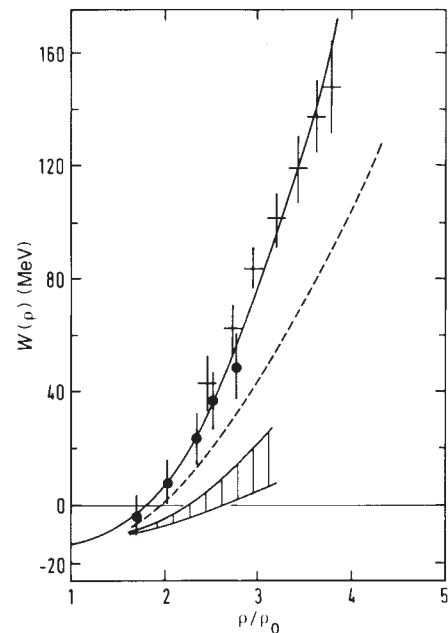


Fig. 6 The energy per nucleon, $W(\rho)$, can be obtained in a semi-empirical way by interpreting various observations with the help of simple theories. The figure shows the first generation of such estimates for $W(\rho)$, with ρ plotted against ρ_0 , the ground-state nuclear-matter density. Heavy-ion collisions indicate a stiff response to compression (data points from pion data²⁰ (crosses) and radial energies²⁴ (circles), and VUU (solid) curve^{25–27}) but the BCK model of supernova dynamics (hatched curve) demands a soft equation of state and indications from heavy neutron stars²⁸ (dashed curve) fall in between. These apparent contradictions may be due both to failings in the reaction models and to real differences between the dynamical conditions, such as temperature, in the different cases.

‘cold’ compressional energy. Our simplification (4) needs modification. Even with a correct model for the thermal energy $W^{\text{therm}}(\rho, T)$, the remaining fraction of the internal energy is not $W^{\text{comp}}(\rho, T=0)$ but a temperature-(velocity)-dependent $W^{\text{comp}}(\rho, T)$. First estimates of this effect indicate a considerable dynamical stiffening of the W function³¹. At $T=100$ MeV and $\rho=4\rho_0$, $W^{\text{comp}}(\rho, T)$ might exceed $W^{\text{comp}}(\rho, T=0)$ by 50 MeV. Thus the neutron star curve in Fig. 6 more probably represents the true $W^{\text{comp}}(\rho, T=0)$ and there are good reasons why the heavy-ion results are well above it.

● Theories have treated thermal degrees of freedom only to first order, ignoring possible shifts of nucleon, pion and resonance masses, and interaction cross-sections inside dense hadronic matter^{32–34}. These ‘effective masses’ could increase the thermal part of the pressure, so that less static compressional pressure would be required to reproduce the sideways flow (Fig. 5). This might lower the VUU curve of Fig. 6 considerably.

● The prompt supernova-bounce model is by no means unchallenged^{35–37}. There are many essential parameters in the physics of the simulation besides the equation of state that are not known with certainty, such as iron core mass, core entropy and degree of neutronization (the neutron concentration in the dense core). An alternative model^{35,36} explains the supernova as a two-step process: the initial shock wave is allowed to stall at about 100 km radius but it is released again by reheating caused by absorption of a neutrino shower emitted by the neutronizing core. In this model stiffer equations of state than in the BCK model are admissible, but it remains to be shown that the energy release of the indirect mechanism suffices to account for the most recent supernova SN1987A.

The confrontation of data from nuclear experiments and astrophysics will obviously prompt further theoretical and experimental work, and the apparent contradictions may in the long run help to solve the puzzle of high-density hadronic matter.

Results from GSI's new heavy-ion synchrotrons will extend the work of LBL's Bevalac, but another nearby supernova would also be useful.

Outlook for quark matter

Whatever refinements and corrections to the equation-of-state data might be made in future, the smooth monotonic increase of $W(\rho)$ is unlikely to disappear. There is no indication yet of a phase transition, for up to about four times the ground-state density or for temperatures up to 100 MeV, where the total energy density amounts to $\sim 0.7 \text{ GeV fm}^{-3}$. Lattice-gauge QCD, which suggests that hadronic matter is stable up to $2\text{--}3 \text{ GeV fm}^{-3}$, was first developed to study the quark-gluon wavefunctions of isolated hadrons, which have an energy density of $\sim 0.5\text{--}1.0 \text{ GeV fm}^{-3}$, and was later adapted to study large volumes and high temperatures^{8,38,39}. What accelerator energy is needed to match the critical conditions for the deconfinement transition from hadronic to quark-gluon matter? The step from 0.7 to 2.5 GeV fm^{-3} at first seems to demand a factor of about four in the centre-of-mass kinetic energy over and above the 0.4 GeV per nucleon attainable at the Bevalac, but at much higher energies we may not be able to count on the nuclear stopping power to accomplish complete thermalization, as observed in the Bevalac (Fig. 3). For example, at the energy of 32 GeV per nucleon obtained for the CERN Intersecting Storage Rings, a mere 15% of available centre-of-mass energy is thermalized in proton-proton collisions. Nucleus-nucleus collisions should provide greater stopping power because of sequential interactions in the extended medium, but we expect to need a twentyfold rather than a fourfold increase in the centre-of-mass energy, that is, $8\text{--}10 \text{ GeV}$ per nucleon.

Because the CERN SPS could yield nuclear projectiles at 200 GeV per nucleon laboratory energy, which corresponds to 9.6 GeV per nucleon in the centre of mass, it was equipped with an injector for heavy ions by a collaboration between GSI, LBL and CERN. Using ^{16}O and ^{32}S projectiles, a set of pilot experiments were run to explore the new territory. The total beam energies of 3.2 TeV for ^{16}O and 6.4 TeV for ^{32}S set the world record for accelerator beams. The detectors are swamped with particles created in high-multiplicity events, as shown for a central collision of ^{16}O and Pb at 200 GeV per nucleon beam energy⁴⁰ (see panel on page 319). This event was recorded in the streamer chamber. About 280 charged particles are created in the collision, chiefly pions, kaons and protons. With an average spatial resolution of 1.5 mm , the total chamber volume of $2 \times 1.2 \times 0.7 \text{ m}^3$ represents a potential information content of about 5 megabytes, enough to deal with multiplicities up to 500, such as those created in central interactions of ^{32}S and Au .

At CERN, a variety of techniques beside the streamer chamber are used to deal with this wealth of information: calorimetry to measure average energy-flow distributions, spectrometers for recording a small subfraction of the created particles at high

precision, and some detectors that are set up specifically to find muon pairs or photons. Similar experiments have been conducted at the Brookhaven AGS synchrotron, with ^{16}O and ^{28}Si beams of 14.5 GeV per nucleon. Recent discussion of the first results from these new experiments indicates that the essential criteria for the formation of the quark-gluon matter are being met. Estimates⁴¹ of the energy density reached in central collisions of ^{16}O and ^{32}S projectiles with heavy target nuclei are $\sim 2.5\text{--}3 \text{ GeV fm}^{-3}$. This meets the critical deconfinement conditions predicted by lattice QCD, and shows that the nuclear stopping power is still effective at 200 GeV per nucleon. Pion correlation data demonstrate that a large, thermalized fireball volume with long lifetime is created at the centre of mass of the collision⁴². Furthermore, there is a suppression of the J/ψ charm-meson production in central ^{16}O and ^{32}S collisions with U nuclei⁴³, which agrees with the prediction for charmonium production from deconfined matter⁴⁴.

Other data, on transverse momentum spectra, strange-particle production, photon production and total multiplicity are currently being examined for indications of new physics. Can they be understood by a simple reaction mechanism, such as cascading of standard nucleon-nucleon collisions, or do they require a different production mechanism which might bear the fingerprints of quark-matter production in the transient, high-density stage? A direct and unambiguous signature of quark-matter formation is still an elusive goal that awaits more theoretical work.

The study of $\text{GeV-per-nucleon-energy}$ heavy-ion collisions has brought within our reach the equation of state for nuclear matter at densities relevant to supernovae and neutron stars. It has also added a new challenge to nuclear many-body theory by introducing relativity and a search for new dynamical models. Looking ahead, to increases in energy by factors of a hundred, the search for collective quark deconfinement is taking shape, with discussions of suitable physics probes and instrumental development. The origin and nature of confinement is still an uncertain part of QCD, well proven only at short ranges ($\sim 0.1 \text{ fm}$), whereas confinement and deconfinement are intermediate-distance (0.5 fm) phenomena. Beside its fundamental interest, the existence and properties of a quark-gluon plasma state would be helpful in understanding of Big Bang expansion.

Until now, the two new research fields have not required a dedicated accelerator or large-scale experimental construction. GSI at Darmstadt is, however, now building a set of second-generation accelerators dedicated to nuclear-matter research. And experimenters at both CERN and Brookhaven are discussing the prospects for heavier projectiles, up to lead. Higher energies might follow later from the Large Hadron Collider project at Geneva, or the proposed Relativistic Heavy Ion Collider at Brookhaven. The analysis of the new data obtained with ^{32}S and ^{28}Si beams at CERN and Brookhaven will soon provide a firmer justification and direction for such developments.

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Geophysical evidence for 'thick-skinned' crustal deformation in central Australia

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Until now, there has been no clear evidence from deep seismic reflection profiles of crustal faults that displace the Moho. New data from central Australia show such a structure in a region characterized by large thrust structures, large gravity anomalies and abrupt changes in teleseismic travel times. Here the principal thrust, the Redbank Deformed Zone, cuts through the crust, and the Moho is significantly displaced across it. The geological and geophysical evidence leads to a model of thick-skinned crustal deformation which occurred in an intra-cratonic environment in late Devonian time.

A CURRENT controversy in the study of continental tectonics is whether deformation initially involves the entire crust and the upper mantle (that is, a 'thick skin') or whether it is controlled by detachment zones within the crust so that much of the folding and imbricate thrusting is restricted to the upper crust alone (a 'thin skin')¹. This controversy has been introduced recently to attempts to understand the formation of the Pyrenees of Europe, for which both thick- and thin-skinned interpretations have been proposed². Well-known examples of thin-skinned tectonics include the Appalachians and Rocky Mountains of the United States and the Alps of Europe. Thick-skinned models are characterized by major planar crustal faults which cut the entire crust at moderately steep angles. Examples of thick-skinned tectonics include the Wind River Thrust of North America, the Grenville Front region of Canada and the Flannan and Outer Isles Thrusts of Great Britain. The Grenville Front in particular is an excellent example of thick-skinned tectonics³. Another example occurs in central Australia, and exhibits clear evidence of a major offset in Moho depth across the structure.

The tectonic setting of central Australia is characterized by a series of late Proterozoic–Palaeozoic basins and an exposed geological basement of middle Proterozoic age^{4–7}. The basins include the Amadeus and Ngalia Basins, and the principal region of exposed basement is the Arunta Block (Fig. 1). The region as a whole is characterized by relatively flat topography and by major east–west-trending gravity anomalies (of peak-to-trough amplitudes exceeding $1,400 \mu\text{m s}^{-1}$ (140 mgal)) (Fig. 1). Teleseismic travel-time anomalies of up to 1.5 s have also been recorded across the region over distances of a few tens of kilometres⁸. Major east–west-trending thrust zones occur within the Arunta Block. These observations, together with the geological evidence that the thrusts were last reactivated in an intra-cratonic environment in late Devonian to early Carboniferous times⁹ with little subsequent tectonism, makes this basin-and-block structure quite unusual and raises a number of fundamental questions. What is the deep crustal structure beneath these basins and blocks? What were the driving forces that shaped these and other structures? How has this anomalous structure been supported since the time of the last major thrusting event¹⁰? Several models have been proposed in response to such questions^{9–12}, and a 500-km-long north–south deep-seismic-reflection traverse has been recorded by the Bureau of Mineral Resources across the Amadeus Basin and Arunta Block¹³. Only the northern section, extending from north of the Ngalia Basin to a point south of the major thrust zone (the Redbank Thrust or Redbank Deformed Zone), is discussed here (the section A–A' in Fig. 1). Explosive sources were used throughout and the resulting deep-seismic-reflection data pro-

vide excellent resolution of the crustal structure down to an average depth of ~40 km.

Regional geology

The Amadeus Basin⁴, bounded to the north by the Arunta Block and to the south by the Musgrave Block, contains up to 14 km of sediments of late Proterozoic (~900 Myr) to late Palaeozoic (~300 Myr) age. The maximum thickness of sediments occurs at the faulted and upturned northern margin, where the Arunta Block has been thrust up and over the basin sediments to form the Amadeus Homocline. Several deformation events have been identified within the basin sediments, with the major and final one having occurred at the time of the Alice Springs orogeny in the late Devonian to early Carboniferous. The northern margin of the Ngalia Basin is also fault-controlled and the last major orogenic event (the Mt Eclipse orogeny) occurred at about the same time as the Alice Springs orogeny⁵. The Ngalia Basin is not an important feature in the seismic-reflection section discussed here because the sediment thickness does not exceed 1 km. Further to the west the sedimentary section exceeds 6 km in thickness.

The Arunta Block can be characterized by three tectonic provinces^{6,7}, each of which underwent separate histories of deformation and metamorphism during early to middle Proterozoic time (Fig. 1). The Southern Arunta Province, between the Redbank Thrust and the northern margin of the Amadeus Basin, is predominantly an amphibolite-grade granitic gneiss, in contrast to the central province which is characterized by felsic and relatively minor mafic granulite that was metamorphosed at depths of ~25 km at ~1,750–1,800 Myr. The Northern Arunta Province consists of low-grade metasediments of amphibolite and greenschist facies. The southern and central provinces are separated by the Redbank Thrust, a 7–10-km-wide east–west-trending zone of anastomosing mylonites which dips northwards at about 45°. The northern and central provinces are separated by large granitoid intrusions and possibly by moderately to steeply dipping faults. Another major structural feature is the Ormiston Thrust Zone some 13 km south of, and running sub-parallel to, the Redbank Thrust. At the northern end of the line, north of the Ngalia Basin, the tectonics becomes complicated by an oblique lineament known as the Weldon Tectonic Zone^{5,6}. Apart from this last feature the tectonic strike is primarily east–west and approximately orthogonal to the seismic reflection line.

Deep seismic reflections

All signal processing was carried out using pseudo-true-amplitude techniques, in which energy loss is assumed to occur only

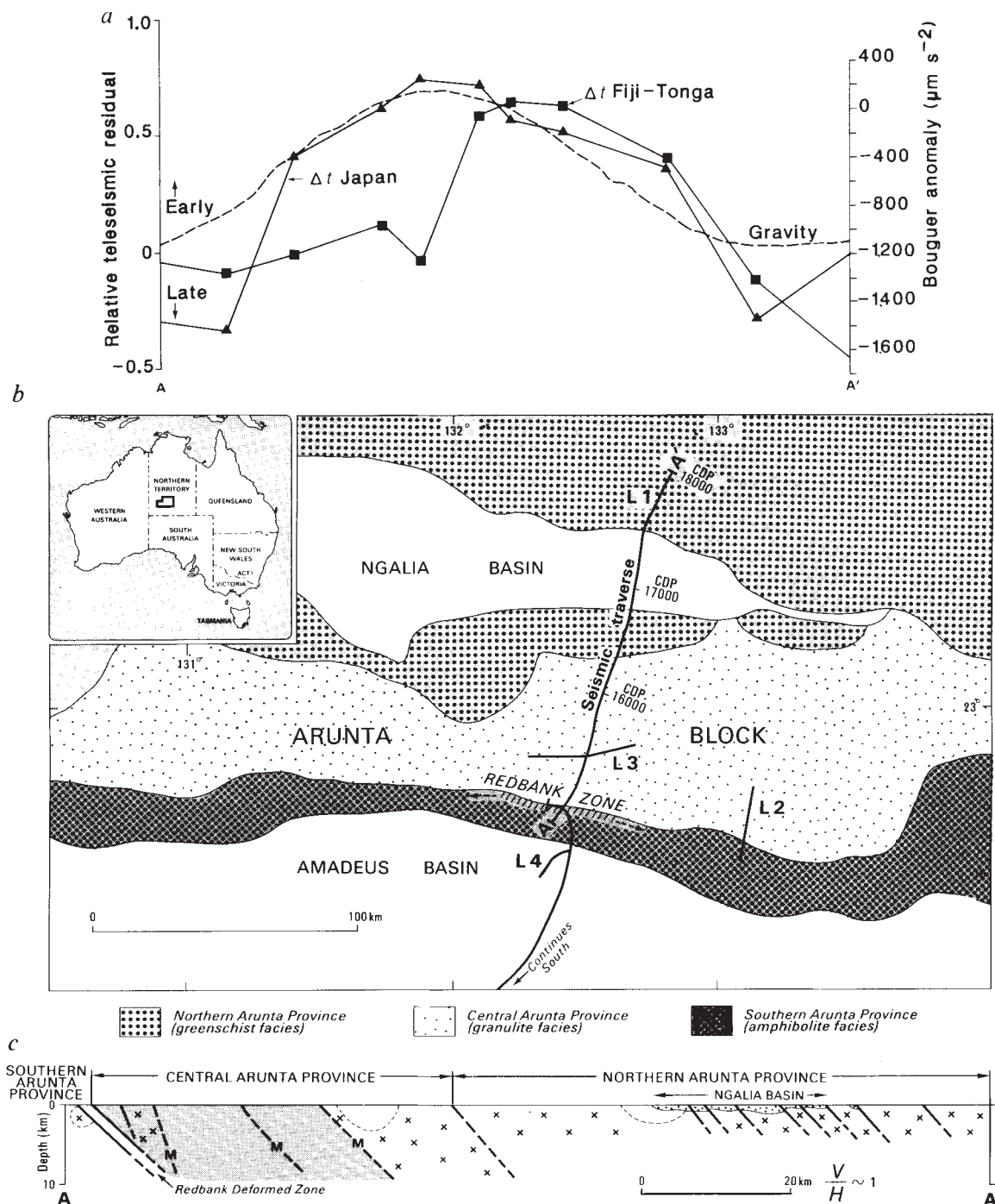


Fig. 1 Location diagram showing the principal tectonic elements of the central Australian region (b) and the position of the northern portion of the Central Australian traverse, L1, where it crosses the Arunta Block. Gravity and teleseismic travel-time profiles¹ (a) and a schematic geological cross-section (A-A', shown in b) along this traverse are also shown (c). The shading schemes used in b and c are different. The travel-time anomalies are for earthquakes from two different regions: Japan from the north and Fiji-Tonga from the east.

by spherical divergence and losses associated with attenuation are neglected. Major efforts to enhance the deep seismic signal were not required during processing. Figure 2 illustrates the variations in the processed signal throughout the seismic section for a collection of single-shot gathers.

Line drawings of the deep-seismic-reflection section are shown in Fig. 3 and are used here instead of seismic-reflection sections because of the greater clarity of the former and to avoid some of the difficulties of migrating deep seismic data¹⁴. Each line segment in these drawings represents a coherent seismic reflection seen on the original seismic section. Results obtained with different stacking methods are illustrated for both unmigrated and migrated sections. The migrated sections are

produced by migrating each line segment of the line drawing to its time-depth position. The coherency-stacking method (Fig. 3a) enhances coherent and continuous dipping and flat reflectors. The corresponding section of migrated line segments is shown in Fig. 3b. The energy-stacking method (Fig. 3c) emphasizes packets of energy that are not affected by signal polarity, and enables an easier correlation of reflections because they are enhanced above the generally noisy background of the seismic sections¹⁵. The corresponding line-segment-migrated section is shown in Fig. 3d. Both migrations were performed with a velocity-depth function that varied from 5.5 km s⁻¹ at shallow depths to 6.2 km s⁻¹.

Other stacking methods have also been used, and they produce

the same general features illustrated in Fig. 3. In addition, most of the deep-reflection events seen on these processed sections can also be identified on the single-fold displays (see, for example, Fig. 2).

The principal features of the line drawings are illustrated in Figs 3 and 4. A general feature of the data is that the strength and depth of penetration of the reflected signal is unusually high for deep seismic data; the signal is still strong to reflection times of 14 s with an unusually high frequency content. Frequencies up to 80 Hz at reflection times of 5–6 s and to 60 Hz at 6–10 s are common, particularly within the Northern Arunta Province. Because conditions around the source were similar to those encountered elsewhere in Australia, the high-frequency content implies that the rocks at depths of 15–20 km have a very high quality factor Q (that is, low attenuation)¹⁶.

The Precambrian Arunta Block exhibits reflections of varying strength throughout the section and is not characterized by the marked non-reflective upper-crustal zone that is seen in similar sections of Precambrian regions elsewhere in the world¹⁷. Instead, the Arunta section is dominated by northerly-dipping reflections throughout the entire crust, which coincide with surface evidence of thrusts and faults. The surface faults and mylonite zones corresponding to the Redbank Thrust correspond to reflections in the seismic section that can be traced to depths of at least 30–35 km and possibly down to 50 km (Figs 3b and 4a), dipping at an angle of about 35–40°. Three-dimensional modelling has shown that such faults can generate strong reflected signals¹⁸. A second band of northerly- but shallower-dipping reflections lies below this feature and its extension to the surface suggests that it corresponds to the Ormiston Thrust, which appears to merge with the Redbank Thrust at mid-crustal levels. Beneath the Southern Arunta Province, the reflectors down to 30 km depth are predominantly sub-horizontal, in contrast to the generally northerly-dipping reflectors (~35°) beneath the central and northern Provinces. This transition in dip coincides with the Redbank Thrust. We interpret the Southern Arunta Province as extending to the underthrust foot wall of this thrust, down to a depth of at least 30 km. The crust north of the Redbank Thrust exhibits further moderately dipping (35–40°) reflections which correspond to surface outcrops of mapped faults or of faults inferred from aeromagnetic surveys. These faults and shear zones extend down to a depth of ~35 km and divide the crust into a series of 'blocks', each of which has similar seismic characteristics and which is bounded by the dipping faults. Seismic-reflection sections of Precambrian crust recorded elsewhere in the world have not exhibited such large numbers of dipping faults and inferred tilted blocks. The shallow zone of low reflectivity (<2 s travel time) seen to the north of the Redbank Thrust coincides with mapped outcrops of granitoid and granitic gneiss, and this whole region is interpreted as granitic material. Sub-horizontal reflections occur at the base of this granitic zone, as well as within the crustal blocks (Fig. 3b), and are attributed to compositional layering. They can, therefore, be used as marker horizons which indicate the present attitude of the original crustal layering. Within some blocks these sub-horizontal events are truncated against the dipping faults, suggesting that the deformation of the crust occurred by movement along the faults, with little or no rotation of the crustal blocks.

Deep-crustal reflections in the migrated sections are more prominent beneath the northern and southern provinces than beneath the Central Arunta Province (Figs 3b, d and 4a). In particular, the two migrated sections beneath the central province exhibit a relatively transparent zone at about 25–30 km depth, where the density of reflections is comparable to those usually seen in other sections at depths greater than 35–40 km. Energy is received from reflections beneath this transparent zone, indicating that this region is seismically non-reflective rather than being a region of high attenuation. The continental crust-mantle boundary is often defined in seismic-reflection

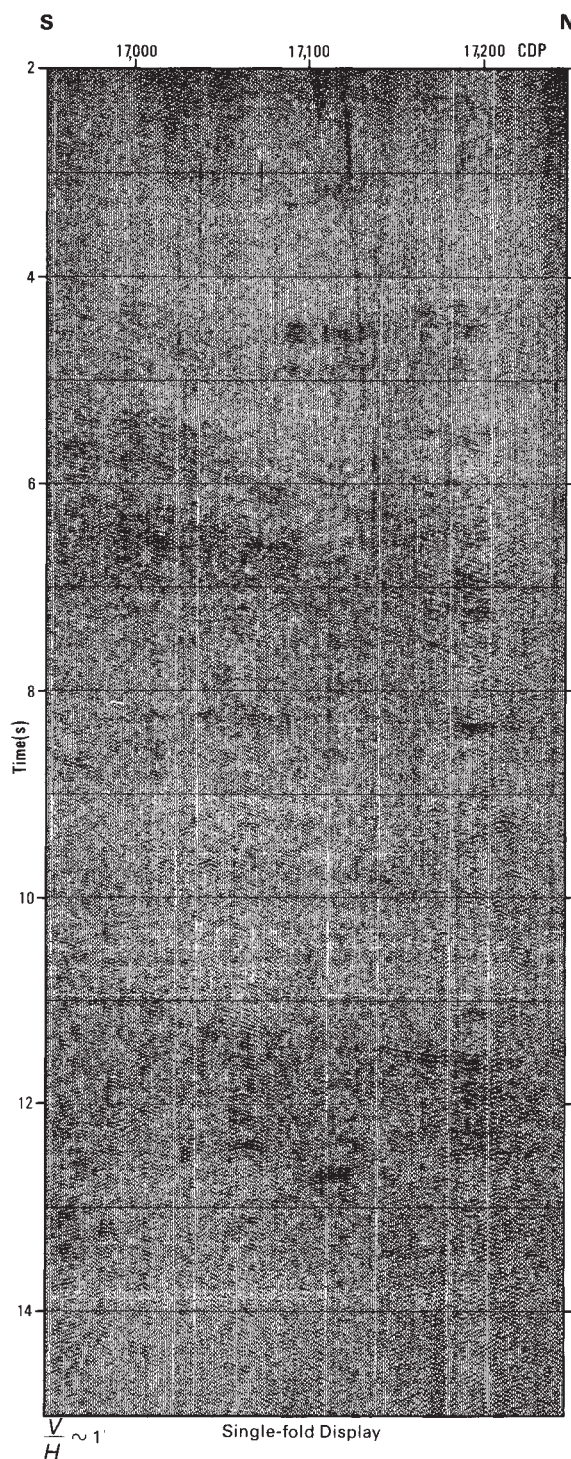


Fig. 2 Portion of a single-fold display, without gain correction, from within the Northern Arunta Province. This is a pseudo-true-amplitude section and illustrates the strength and continuity of the deep signals.

sections as the base of a zone of strong, deep and layered reflections overlying a non-reflective region corresponding to the upper mantle^{19,20}. Beneath the northern part of the Arunta Block, the appropriate choice for this boundary would be at about 35–40 km depth but it is less well defined south of the Redbank Thrust. One interpretation is that this boundary occurs at the base of the lowest reflections that are seen at about 50 km depth (Figs 3b and 4a); this indicates a thicker-than-normal crust and a significant variation in the crustal thickness between the southern and northern provinces. Sediments in the Amadeus

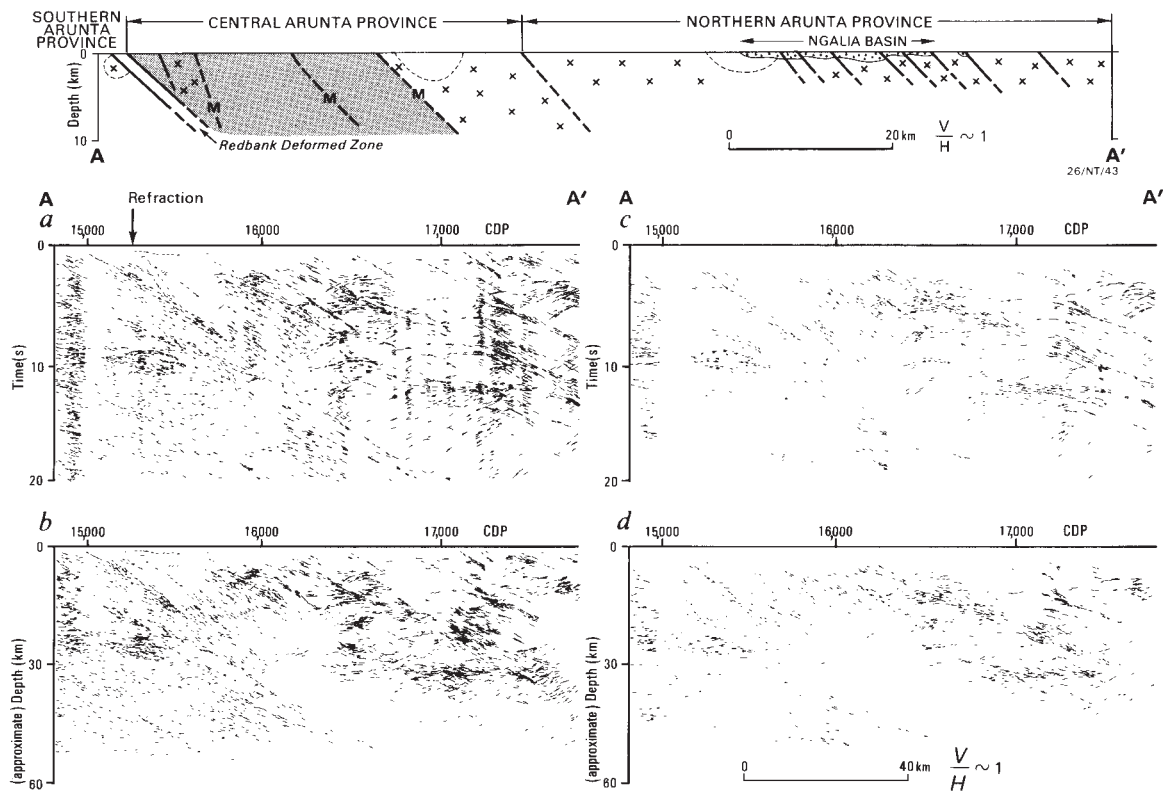


Fig. 3 Unmigrated and migrated line drawings of the seismic-reflection section from the Central Australian traverse. *a*, Unmigrated coherency stack. *b*, Line-migrated version of *a*. *c*, Unmigrated energy stack. *d*, Line-migrated version of *c*.

Basin, south of the Redbank Thrust, have a maximum thickness of 10–14 km and the gravity, teleseismic travel-time anomalies and deep-crustal-reflection data do not indicate any discontinuity in the deep-crustal structure across the basin margin; the interpretation that the base of the crust is at 50 km depth therefore implies that the sediments have been deposited on a crust of approximately normal thickness. This is consistent with a predominantly compressional regime during the later part of the basin formation process. The large negative gravity anomalies, the late teleseismic travel-time arrivals (Fig. 1) across the northern part of the Amadeus Basin and the southern part of the Arunta Block south of the Redbank Zone, and an east-west seismic reflection profile, all favour a crustal thickness of more than 50 km in this region. We therefore interpret the reflection record beneath the footwall of the Redbank Thrust as a broad crust-mantle transition zone of 15–20 km thickness extending down to a depth of at least 50 km. Beneath the Northern Arunta Province this transition zone is considerably thinner and occurs at a shallower depth. The seismically transparent zone immediately north of the Redbank Thrust is interpreted as a region of predominantly mantle material emplaced by major thrusting on the Redbank and Ormiston Thrusts.

Figure 4b illustrates the crustal structure derived from the teleseismic travel-time anomalies for the part of the Arunta Block corresponding to the extent of the seismic line. The model has poor resolution north of the Ngalia Basin. The principal requirements of this data set are a zone of relatively high-velocity material dipping steeply northwards such that its continuation to the surface coincides approximately with the Redbank Thrust and the granulite terrain to the north of it, and relatively low-velocity material in the form of a down-thrust wedge to the south of the Redbank Thrust¹⁰. These features are consistent with the reflection data.

Gravity modelling

The Bouguer gravity anomalies along the seismic line are characterized by a relatively long-wavelength variation of

$\sim 1,400 \mu\text{m s}^{-2}$ peak-to-trough amplitude. The free-air anomalies exhibit a very similar variation, and conventional models of isostatic compensation fail to explain these observations²¹. Near-surface variations in crustal densities of surface outcrop rocks range from 2.5 g cm^{-3} for the Palaeozoic sediments in the basin to 2.95 g cm^{-3} for the high-grade granulite facies rocks north of the Redbank Thrust¹⁰. If such lateral variations persist down to 10 km depth they would explain about 50% of the observed magnitude of the gravity anomalies. However, the wavelength predicted for these anomalies is considerably less than that observed, and the observed maximum anomaly is offset from the surface exposure of high-density granulites by more than 50 km. Furthermore, there are no significant short-wavelength changes observed across either the Redbank Thrust or the Amadeus Homocline, despite the existence of density contrasts across these surfaces¹⁰. Overall, these observations suggest a deep origin for the major part of the observed gravity field over the southern part of the Arunta Block. Figure 5 shows the gravity field predicted by the model, using density values based on surface-rock exposure^{9,10}. Agreement with the observed field is satisfactory and could be further improved by minor adjustments to the boundaries that separate regions of different densities or by introducing density gradients within the regions. Most importantly, the model makes good predictions of both the amplitude and the wavelength of the observed field.

A tectonic model for the Arunta Block

The deep-seismic-reflection data favour a tectonic model in which the major deformation within the Arunta Block occurred along faults and shear zones that extend from the surface to the crust-mantle boundary (that is, a 'thick-skinned' model). In particular, there is no seismic evidence for mid-crustal ramps branching from a relatively shallow-dipping sole thrust, which is a principal characteristic of the thin-skinned models²². In this sense, the Arunta structure is comparable to both the Flannan and Outer Isles Thrusts of Scotland²³, the Pyrenees of Europe²,

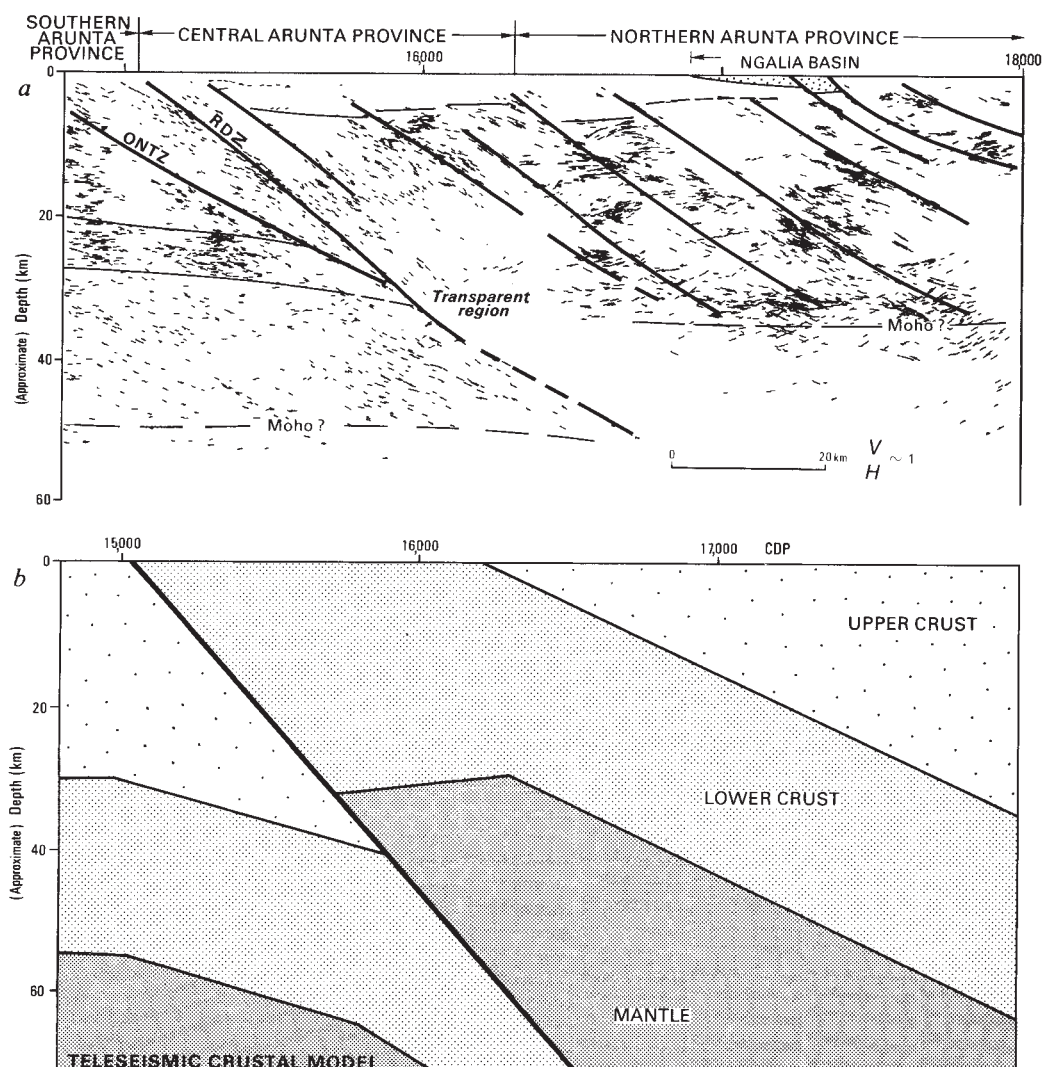


Fig. 4 Interpreted crustal structure of the Arunta Block. RDZ refers to the Redbank Deformed Zone and ONTZ to the Ormiston Nappe and Thrust Zone. *a*, Deep-seismic-reflection model. *b*, Teleseismic crustal model, based on the observed teleseismic travel-time data¹.

the Grenville Front of Canada⁵ and, to a lesser degree, the Wind River Thrust of the USA²⁴, but it differs in that these latter structures do not exhibit the seismically transparent material on the lower part of the hanging wall. None of the Flannan, Outer Isles or Wind River Thrusts are associated with a major gravity anomaly. It is emphasized that the above remarks refer to the central part of the Arunta Block and Amadeus Basin, and that in an intracratonic environment the deformations along the eastern and western limits may differ in significant ways from this model.

Structural, metamorphic, and isotope data reveal that the Arunta Block has had a complex history. In particular, Rb/Sr and ⁴⁰Ar/³⁹Ar isotope data imply different evolutionary histories for the blocks north and south of the Redbank Thrust before 1,450 Myr and possibly until 1,100 Myr (ref. 9). The overall character of the seismic reflections changes across this thrust and this may be indicative of fundamental differences in crustal composition of the two regions, supporting the interpretation that the Redbank Thrust is a major crustal boundary. Furthermore, the isotope data and the metamorphic grade of the mylonites indicate that the granulites north of the Redbank Thrust were subjected to major uplift (~15 km) during the Alice Springs orogeny, particularly during the last phase of the Amadeus Basin formation which produced the upturned and overthrust northern margin (the Amadeus Homocline). Argon-loss data constrain this uplift at between 300–400 Myr, with the movements on the faults decreasing in age from north to south between the Redbank and Ormiston Thrusts⁹.

Teleseismic travel-time residuals¹⁰ favour models in which

low-velocity crust is underthrust beneath overriding lower crust and mantle of the Central and Northern Arunta Provinces down to a depth in excess of 50 km (Fig. 4). The isotope evidence shows that a substantial part of this structure developed at a time leading up to and including the Alice Springs orogeny, producing a stacking or imbrication of the crust and the exposure of lower-crustal material north of the Redbank Zone. Displacement of the inferred granitic layer and the deflection of sub-horizontal reflectors against the fault or shear zones suggest that the faults north of the Redbank Thrust acted as 'lag' faults during the final stages of movements.

This model generates a number of questions. In particular, one can ask what are the driving forces that have produced this structure, how such a structure, obviously out of isostatic equilibrium, has been maintained for the past 300 Myr, and how these conclusions relate to other thick-skinned regions. The stress differences associated with the present structure are of the order of 100–150 MPa (ref. 10) and the stress fields associated with the forces that produced the structure must have been at least of this magnitude. The isostatic response is anisotropic. The east-west response is consistent with models of local or regional isostatic compensation, whereas the north-south response implies compressional in-plane stresses of the order mentioned²¹. The major movements on the Redbank Thrust before and during the Alice Springs orogeny indicate that compressional forces operated at this time, and the geological setting of central Australia indicates that this occurred within an intracratonic environment rather than at a plate margin. The timing of the Alice Springs orogeny coincides with the Kanimblan

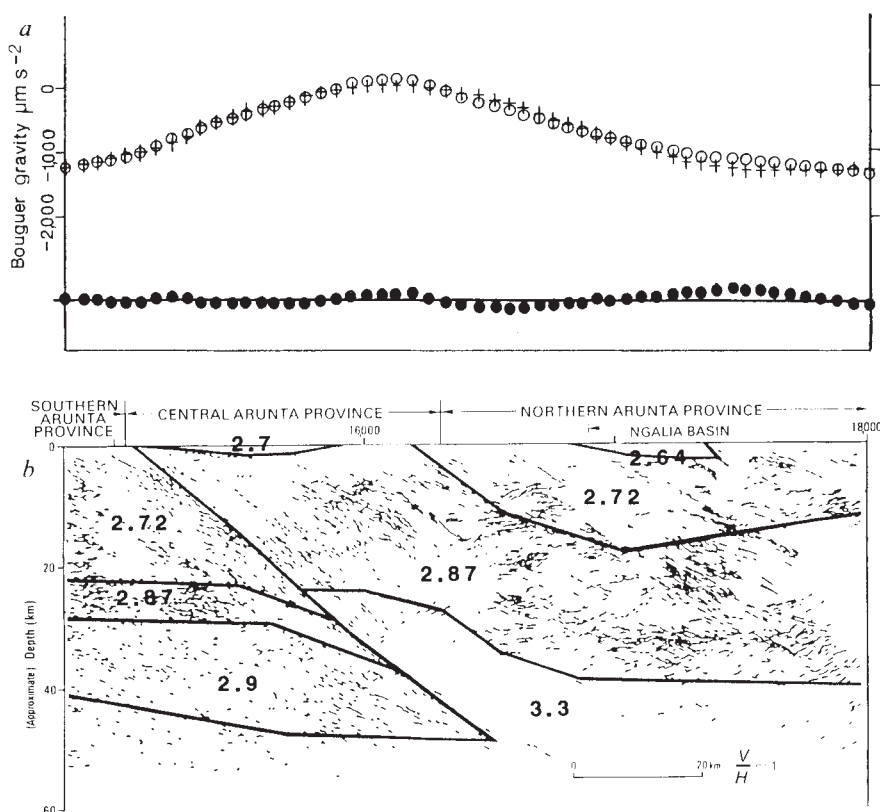


Fig. 5 a, Observed and computed gravity profiles along the deep seismic line. The computed profile is based on the interpreted deep-seismic-reflection model (b) for the Arunta Block. In a, crosses represent the observed profile, open circles represent the computed profile and filled circles represent the residual profile $\pm 15 \mu\text{m s}^{-2}$. Numbers superimposed on b are the densities in g cm^{-3} assumed in the gravity calculations.

orogeny of eastern Australia²⁵; this suggests that the intracratonic movement may have been primarily the consequence of the lithosphere acting as a stress guide to forces operating at and shaping the plate margin some 1,000 km or more distant from the Redbank Zone²⁶. The implication is that stress differences of 100 MPa and more can be transmitted by the lithosphere over distances equal to the dimensions of tectonic plates and that the evolution of intracratonic tectonic features such as basins may be driven by forces external to the area. Evidence for this can also be seen elsewhere, for example, in the relation between the tectonics of north-west Europe and the latest Cretaceous and Tertiary Alpine orogeny²⁷.

Once formed, stress relaxation would be expected to lead to isostatic rebound so that the area would reach some state of mechanical equilibrium. There is, however, surprisingly little evidence for major re-adjustment with time. The maximum gravity anomaly is associated with a very mild topographic low and the minimum anomaly is associated with a regional topographic high over the northern part of the basin, suggesting that some rebound has occurred¹⁰. At the time of the last orogenic event the area would have carried a significant topographic load created by the upthrust crust and by the synorogenic sediment loads deposited on the flanks of the thrust. A study of fission-

track ages²⁸ and isotope evidence⁹ points to a removal of up to 4 km of material from both the northern part of the Amadeus Basin and the southern part of the Arunta Block. Thus, immediately after the orogenic event, the region may have been close to isostatic equilibrium, but as erosion occurred the crust did not respond fully to the redistribution of surface loads. The probable explanation is that part of the north-south horizontal compressive force that led to the thrusting was still present after termination of the orogeny, at which time the lithosphere strengthened, locking the sub-surface structure and producing an increasingly regional response to the lateral variations in surface and internal loads²². We note that two major earthquake sequences occurred recently in central Australia, one to the south, in the Musgrave Block²⁹, and the other about 350 km north of the Ngalia Basin³⁰, both of which indicate that the present lithospheric stress field is one of north-south compression.

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A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis

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The telomerase enzyme of Tetrahymena synthesizes repeats of the telomeric DNA sequence TTGGGG de novo in the absence of added template. The essential RNA component of this ribonucleoprotein enzyme has now been cloned and found to contain the sequence CAACCCCAA, which seems to be the template for the synthesis of TTGGGG repeats.

NUCLEIC acid synthesis reactions are usually template directed. Exceptions include the reactions of terminal deoxynucleotidyl transferase, which adds any deoxynucleotide in a random order onto the 3' end of a polynucleotide chain¹, poly(A) polymerase which adds rAMP residues to the 3' end of RNA², and tRNA nucleotidyl transferase which adds the sequence CCA to the 3' end of tRNAs³. In addition, we have discovered the enzyme telomerase which synthesizes tandem repeats of the telomeric DNA sequence TTGGGG to the 3' end of telomeric primers without any added template^{4,5}.

Telomeres from such diverse organisms as ciliates, yeasts, plants, and mammals are made up of a variable number of tandemly repeated, simple (G+C)-rich sequences⁶⁻¹². In all telomeres examined, there is a strand-specific sequence bias such that the G-rich strand always runs 5' to 3' towards the end of the chromosome. As the number of tandem repeats on the end of any given chromosome is variable^{13,14}, restriction fragments containing telomeres have a characteristic 'fuzzy' appearance in agarose gel electrophoresis. We have proposed that this length heterogeneity arises from a dynamic equilibrium established between the incomplete replication and/or degradation of the chromosome end and the *de novo* synthesis of telomeric sequences by telomerase^{4,12-14}.

The *Tetrahymena* telomerase extends the free TTGGGG-OH 3' telomere strand by the addition of further TTGGGG repeats. Both the repeated sequence motif and the orientation of the G strand have been conserved throughout evolution, suggesting that telomerase-like enzymes may be found in all eukaryotes. The presence of telomerase would explain several observations about telomeres. First, when *Tetrahymena* or *Oxytricha* telomeric sequences are introduced into yeast, telomeric sequences characteristic of yeast are added to them^{12,15,16}. Second, when trypanosomes or *Tetrahymena* are kept in continuous log-phase growth, there is a net increase in telomere length^{13,17}. In *Tetrahymena* this increase involves all of the telomeres in the cell and is due to the addition of telomeric TTGGGG repeats^{13,14}. Finally, telomeric sequences from a number of different organisms are maintained as perfect tandem repeats and do not diverge through recombination, unlike most simple repeated sequences in the nuclear genome¹⁸⁻²⁰ and mitochondrial telomeres^{21,22}. This sequence maintenance can be explained by the continual synthesis of new perfect telomeric repeats.

Recently, telomerase activities have been isolated from the nuclei of both the hypotrichous ciliates *Oxytricha* and *Euplotes* (ref. 23; D. Shippen-Lentz and E. Blackburn, submitted). These enzymes synthesize repeats of the telomeric sequences of those organisms, TTTTGGGG, in an analogous way to the *Tetrahymena* telomerase. Together with the conserved structure of telomeres, these results further indicate that telomerase enzymes exist throughout eukaryotes.

We showed previously that the telomerase of *Tetrahymena* is a ribonucleoprotein (RNP) enzyme with essential RNA and protein components⁵. Because the reactions catalysed by many

RNPs involve base pairing of the RNA components²⁴⁻²⁸, the requirement of an RNA component in the *Tetrahymena* telomerase suggested that the synthesis of TTGGGG repeats might be specified by the RNA⁵. We report here the identification of the essential RNA component of telomerase and show that this RNA contains the sequence CAACCCCAA which could serve as a template for the synthesis of TTGGGG repeats.

Cloning and characterization of 159 RNA

We previously showed that small RNAs of approximately 159 bases and 80 bases co-purified with the telomerase RNP over five column purification steps⁵. Further purification of the telomerase over several other column series indicated that the RNA of about 159 bases (159 RNA) was the only one to reproducibly co-purify with the enzyme activity (data not shown). We directly sequenced the 159 RNA using both sequence-specific RNases and dideoxynucleotide primer extension techniques. Southern blot analysis of *Tetrahymena* macronuclear DNA, using oligonucleotide probes derived from the RNA sequence, indicated that there was a single gene for the 159 RNA in the macronuclear genome. A 2 kilobase (kb) *HindIII* fragment containing the gene was cloned from macronuclear DNA. The sequence of a 400-base region of the clone containing the 159 RNA coding region is shown in Fig. 1a. The most notable feature of the gene is the presence of the sequence 5' CAACCCCAA 3' from positions 43 to 51 within the coding region. This sequence could serve as a template for the addition of TTGGGG repeats *in vitro*.

Three ribonucleotide residues at positions 46, 47 and 48 in the 159 RNA were not cut by any of the ribonucleases used in the direct RNA sequencing, indicating that these bases might be modified, although primer extension by reverse transcriptase was unimpeded at these positions. Several different base modifications in tRNAs are known to interfere with ribonuclease cleavage²⁹. Ribose 2'-O methylation inhibits both RNase and base hydrolysis of RNA by blocking the formation of the cyclic phosphate intermediate³⁰. As the base hydrolysis ladder was complete at these positions in the telomerase RNA (data not shown), this type of modification can be ruled out. Both secondary structure modelling of the telomerase RNA (I. Tinoco, personal communication) and data presented below indicate that this region of the RNA is not in a tightly base-paired structure; hence structural interference was probably not responsible for the inability of RNases to cut at these positions. Although the nature of these possible base modifications is not known, it is intriguing that they lie within the CAACCCCAA sequence.

Several features of the 159 RNA gene suggest that it is transcribed by RNA polymerase III. First, the length of the 159 RNA is heterogeneous. Using high-resolution polyacrylamide gel electrophoresis we separated a cluster of four bands and sequenced the two prominent species in the middle. These two species differed only in the number of U residues at the 3' end. In the sequence of the cloned gene this 3' end of the RNA falls

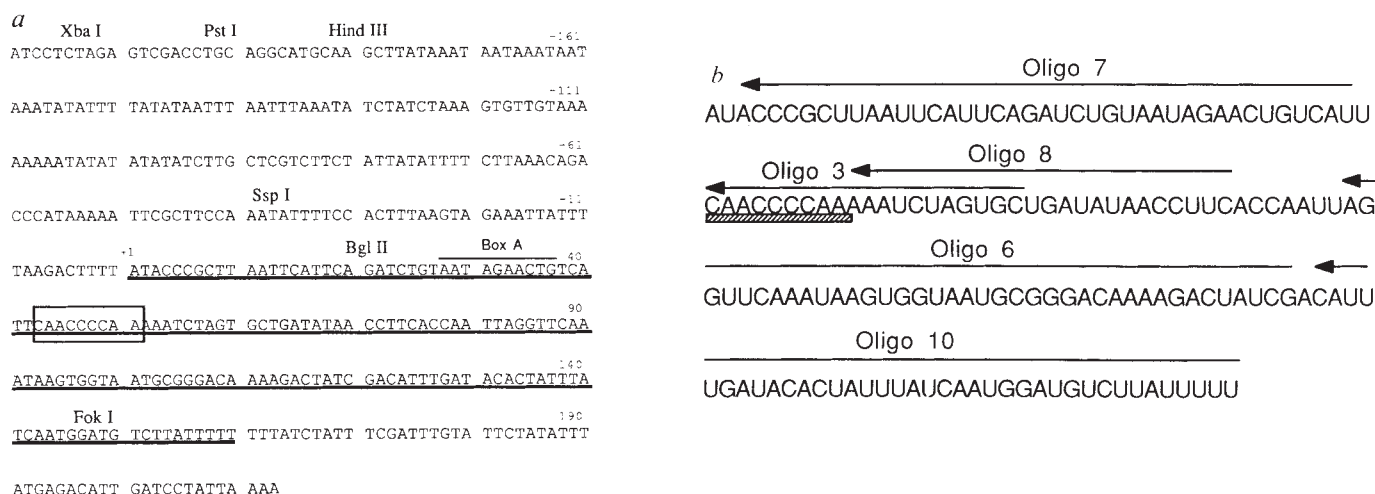


Fig. 1 *a*, Sequence of the telomerase RNA gene. The first 30 nucleotides of the pUC119 polylinker up to the *Hind*III site used for cloning are shown for orientation along with 370 bases of the non-template strand of the telomerase RNA gene. The region corresponding to the RNA is underlined. The start site of transcription as determined by dideoxy primer extension sequencing is labelled +1 and the 3' end of the RNA as determined by direct RNA sequencing is shown within the run of eight T residues near position 160. All of the six-base recognition restriction enzymes which cut in the *Tetrahymena* DNA are shown above their recognition sites. A region of similarity with the consensus Box A for polymerase III genes is shown at positions 28-37. The sequence CAACCCCAA within the RNA gene which may provide the template for the addition of TTGGGG sequences *in vitro* is shown in the box at position 43. *b*, The oligonucleotides (oligos) used for cloning, sequencing and RNase H experiments are shown above the telomerase RNA regions to which they hybridize. The region of the RNA containing the potential template is underlined with a hatched bar. Oligonucleotides 1-6 were made using the sequence information obtained by partial enzymatic RNase sequencing. Oligonucleotides 7, 8 and 10 were synthesized using DNA sequence information. Only the oligonucleotides that were shown to hybridize to the 159 RNA are shown. The sequence and name of all the oligonucleotides used throughout this paper are listed from 5' to 3'. Oligo 2, TTCTGTTGCAAAAATCTGAATGAAT. Oligo 3, GCACTAGATTTTGGGGTTG. Oligo 5, TTGTTTGAACCTGATTGTGAAGGTT. Oligo 6, CGATGGTCTTTTGTCCCGCATTGCCACTTGTGTTGAACCT. Oligo 7, ATGACAGTTCTATTACAGATCTGAATGAATGAATTAAGCGGGT. Oligo 8, GAAGGTTATCAGCACTAGATTT. Oligo 10, AAAATAAGACATCCATTGATAAATAGT-GTATCAAAATG.

Methods. Telomerase S100 preparation (60 ml) was fractionated over a sizing column, heparin agarose, and spermine agarose⁵. An aliquot of the active fraction (30 ml) was digested with proteinase K, phenol extracted and ethanol precipitated; half of the RNA was 3' end-labelled with [³²P] pCp using RNA ligase and half was treated with calf intestinal phosphatase, phenol extracted and labelled with [³²P]ATP using polynucleotide kinase. The labelled RNA was run on an 80 cm 15% acrylamide/7M urea gel for 21 hours at 3,500 V. The two major bands corresponding to the 159 and 160 species of RNA were cut out, eluted from the acrylamide and partial enzymatic RNA sequencing was done. The sequence obtained from the 5' and 3' labelled RNAs overlapped by 50 bases. Thus the entire sequence was obtained except for a few bases where the sequence data from both 5' and 3' labelled RNA was unreadable possibly due to RNA base modifications (see text). Using this sequence information, oligonucleotide 6 was synthesized and used as a primer for dideoxy primer extension RNA sequencing. Eighty-one nucleotides of sequence were obtained and oligonucleotides were then synthesized and used for cloning of genomic copy of the RNA. *Tetrahymena* macronuclear DNA was cut with *Hind*III and size fractionated on a 0.8% agarose gel. The region around 2.0 kb was cut out and electroeluted. This DNA was ligated into *Hind*III-cut pUC119 plasmid and transformed into DH5α F'. 30,000 transformants were obtained: of these 10,000 were screened by colony hybridization. Replica plates were made of the transformation plates, and duplicate colony lifts onto nitrocellulose were made. The duplicate filters were hybridized in 6x SSC, 4x Denhardt's and 0.1% SDS with either oligonucleotide 3 or oligonucleotide 6 at 55°C for 15 hours. The filters were then washed three times at room temperature in 3x SSC 0.1% SDS for 10 min, and then in 3x SSC 0.1% SDS at 55°C for 10 min. One clone was obtained which hybridized to both oligonucleotides. The clone was sequenced using double stranded dideoxy sequencing (USB-sequenase kit) with the reverse universal primer and an oligonucleotide internal to the RNA gene as primers.

in a stretch of eight T residues. These properties are typical of RNA polymerase III transcription termination³¹. Second, we were able to label the 5' end of RNA with [³²P]ATP and polynucleotide kinase after treatment with phosphatase, indicating 5' end of the 159 RNA was not capped (data not shown). Third, there is a region of similarity to the box A consensus RRYNNARYGG³² beginning 30 nucleotides downstream of the 5' end of the 159 RNA.

d(TTGGGG)₄ elongation interference

We used RNase H inactivation to establish whether the 159 RNA was an essential component of telomerase. This technique has been used to identify essential RNAs for a variety of RNPs^{27,28,33-36}. Telomerase was preincubated with antisense oligonucleotides (Fig. 1*b*) in the presence or absence of RNase H. After the preincubation, the primer d(TTGGGG)₄ oligonucleotide was added along with [³²P] dGTP, dTTP and reaction buffer, and the ability of telomerase to synthesize TTGGGG repeats was assayed (Fig. 2). Oligonucleotides 2, 5, 6, 7 and 10, which are complementary to the 159 RNA, had no effect on telomerase activity in either the presence or absence of RNase

H. However, oligonucleotide 3 strongly inhibited telomerase activity in either the presence or absence of RNase H, and preincubation with oligonucleotide 8 resulted in an upward shift in the characteristic six-base banding pattern, suggesting this antisense oligonucleotide was itself efficiently priming repeat addition (Fig. 2; see lanes 17-20). In the absence of added RNase H the 159 RNA was intact, indicating that endogenous RNase H was not responsible for the inhibition of telomerase by oligonucleotide 3 (Fig. 4 and data not shown). Although in this initial experiment the effects of RNase H addition were not definitive, the observations that oligonucleotides 3 and 8, which hybridize across, and near to, the CAACCCCAA sequence respectively, specifically affected telomerase activity suggested the 159 RNA is involved in telomerase activity.

Northern analysis of RNA extracted from active telomerase fractions probed with oligonucleotides 3 and 8, showed only one band at 159 nucleotides (data not shown). Thus, these oligonucleotides do not share extensive sequence complementarity with other RNAs in the enzyme fractions used throughout this work.

The inhibition of telomerase activity by preincubation with

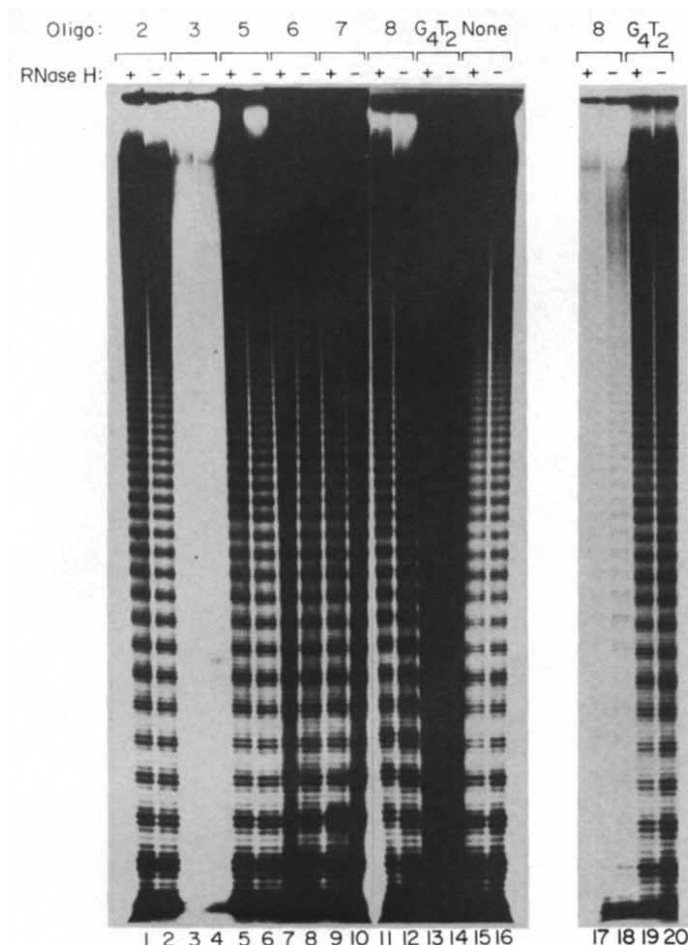


Fig. 2 Treatment of telomerase with antisense oligonucleotides and RNase H. The oligonucleotides indicated were preincubated with telomerase in the presence (+) or absence (-) of RNase H. The ability of telomerase to elongate added d(TTGGGG)₄ primer was then assayed. The oligonucleotides added in the preincubation are indicated above the lanes. G₄T₂ is d(TTGGGG)₄. Lanes 17–20 show a lighter exposure of lanes 11–14 to indicate better the upward shift in the banding pattern when oligonucleotide 8 is included in the preincubation.

Methods. Telomerase which had been purified over a sizing column and heparin agarose was used⁵. Antisense oligonucleotide (2 µl) at 0.4 µg µl⁻¹ was added to 10 µl of telomerase, followed by either 2 µl of RNase H at 2 U µl⁻¹ (BRL) or 2 µl of TMG+0.1 M NaCl (TMG, 10 mM Tris pH 8.0, 1 mM MgCl₂, 10% glycerol) were added. The samples were preincubated at room temperature for 30 min and then 6 µl of TMG+0.1 M NaCl and 20 µl of 2x reaction mix was added. The 2x reaction mix contained: 0.1 µg d(TTGGGG)₄, 200 µM dTTP, 200 mM NaOAc, 100 mM Tris pH 8.0, 10 µCi [³²P]dGTP at 400 Ci mmol⁻¹. Reactions were carried out at 30 °C for 60 min, the mix then phenol extracted, ethanol precipitated and run on a 6% polyacrylamide sequencing gel.

oligonucleotide 3 even in the absence of RNase H suggested that this oligonucleotide might interfere with the access of d(TTGGGG)₄ to telomerase. To test for specific inhibition, we reversed the order of addition of the two oligonucleotides. When oligonucleotide 3 was used in the preincubation and the d(TTGGGG)₄ was added at the beginning of the elongation reaction (referred to as time zero) there was the same inhibition as in Fig. 2. If d(TTGGGG)₄ was added first to the preincubation and then oligonucleotide 3 was added at time zero, there was no inhibition however. When oligonucleotide 3 and d(TTGGGG)₄ were added together, an intermediate level of inhibition was seen (Fig. 3a). Thus, inhibition by oligonucleotide 3 is a specific effect and its properties are consistent with competition with d(TTGGGG)₄.

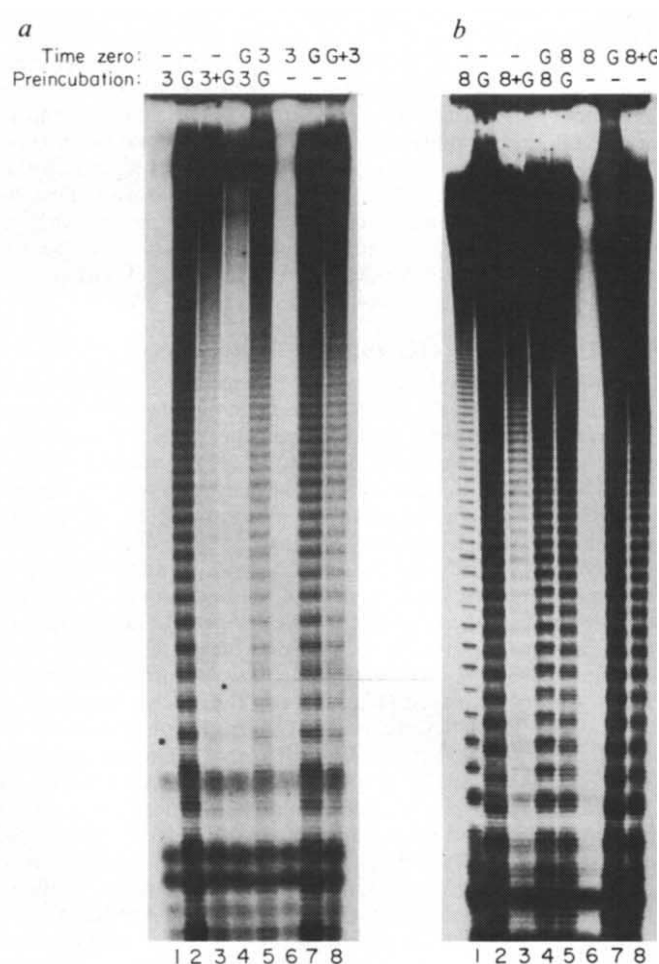


Fig. 3 *a*, Competition between oligonucleotide 3 and d(TTGGGG)₄. Telomerase was preincubated at room temperature with the oligonucleotides indicated above the lanes. G is d(TTGGGG)₄. At time zero, reaction buffer was added and the reaction was carried out as described in the Fig. 2 legend. The oligonucleotides indicated were added at time zero. *b*, Competition between oligonucleotide 8 and d(TTGGGG)₄. The oligonucleotides indicated above the lanes were added in the preincubation. G is d(TTGGGG)₄. At time zero the oligonucleotides indicated were added. Darker exposure of the autoradiogram showed a banding pattern in lane 6 similar to that in lane 1.

Methods. 20 µl of telomerase purified as described in the Fig. 2 legend was preincubated with 1 µl of each oligonucleotide at 0.4 µg µl⁻¹ or with 1 µl of water as indicated. Preincubation was at room temperature for 30 min. At time zero 20 µl of 2x reaction mix containing 1 µl of the appropriate oligonucleotides or no oligonucleotide was added and the standard activity assay was carried out (see Fig. 2 and ref. 5).

As oligonucleotide 3 contains the sequence TTGGGGTTG at its 3' end, its interference with d(TTGGGG)₄ elongation could simply be due to its sequence similarity with the d(TTGGGG)₄ primer. We also found that oligonucleotide 8, which does not contain any TTGGGG sequence, is itself efficiently elongated by telomerase (Fig. 3b). The addition of oligonucleotide 8 alone resulted in the synthesis of a repeat pattern that was shifted upward relative to the pattern primed by d(TTGGGG)₄. The repeat pattern visualized by this gel assay is dependent on the sequence and length of the input primer oligonucleotide⁵. Thus, the upward shift in the banding pattern distinguishes the products primed by addition to oligonucleotide 8 from those primed by d(TTGGGG)₄. When oligonucleotide 8 was added in the preincubation and d(TTGGGG)₄ was added at time zero, the main products had the oligonucleotide 8-primed pattern. In contrast, when the d(TTGGGG)₄ oligonucleotide was added

first and oligonucleotide 8 was added at time zero, the characteristic d(TTGGGG)₄ primed pattern was seen. Simultaneous addition of the two oligonucleotides, either in the preincubation or at time zero, resulted in a mixture of the two patterns indicating that both oligonucleotides were being elongated under these conditions. As oligonucleotide 8 was the only oligonucleotide with a non-telomeric sequence, of 14 tested, that was efficiently elongated by telomerase (see also ref. 5; data not shown), we suggest that the base pairing of oligonucleotide 8 to the 159 RNA adjacent to the CAACCCCAA sequence allows its elongation by telomerase.

Inactivation by cleavage of 159 RNA

The interaction of oligonucleotides 3 and 8 with telomerase *in vitro* suggested that the 159 RNA is a component of telomerase. To investigate further the requirement for this RNA, we sought conditions under which the effects of cleavage of the 159 RNA could be assayed. In RNase H inactivation experiments oligonucleotides 2, 6, 7 and 10, complementary to the 159 RNA, did not inhibit telomerase activity or cause cleavage of the 159 RNA by RNase H (Fig. 2 and data not shown). To assay the effects of oligonucleotide 3 and RNase H, we had first to relieve the inhibition by oligonucleotide 3. We pelleted the telomerase in an ultracentrifuge after preincubation with the test oligonucleotide and RNase H. The enzyme activity of each resuspended pellet was then assayed (Fig. 4a) and the RNA examined for evidence of 159 RNA cleavage (Fig. 4b). There was no effect on either telomerase activity or the 159 RNA after preincubation with oligonucleotide 7 in either the presence or absence of RNase H. After preincubation and subsequent removal of oligonucleotide 3 in the absence of RNase H, both telomerase activity and the 159 RNA were intact. Preincubation with oligonucleotide 3 in the presence of RNase H however, inhibited telomerase activity and caused cleavage of the 159 RNA. In a separate experiment, we used primer extension to map the sites at which RNase H cuts in the telomerase RNP (Fig. 5). Although in this experiment cutting by RNase H was incomplete, oligonucleotide 3 directed cutting at several positions along its region of complementarity to the 159 RNA, including nucleotides in the CAACCCCAA sequence.

Oligonucleotide 8 also directs RNase H cutting of the 159 RNA; cutting with this oligonucleotide, even when apparently complete, did not however inactivate the telomerase (data not shown). Oligonucleotide 8 cannot base pair in the CAACCCCAA sequence and accordingly all of the cut sites mapped by primer extension are 3' to the potential template sequence (Fig. 5). Although the ability of telomerase to function after oligonucleotide 8-directed cleavage of the RNA was surprising, it is not unprecedented. The signal recognition particle retains its microsomal translocation activity after micrococcal nuclease digestion which removes up to 150 nucleotides of the 300 nucleotides in the essential 7SL RNA^{37,38}. In addition, the 25S rRNA from mature ribosomes of insects is found naturally cleaved into two halves with some sequences missing^{39,40}. Furthermore, in *Crithidia*, the 25S RNA is found cleaved into five pieces⁴¹ and in *Chlamydomonas* both the large and small rRNAs are processed into multiple pieces that are held together by intermolecular base pairing⁴². Finally, deletion and replacement analysis of the essential RNA component of the yeast U2 small nuclear RNP has shown that not all regions of this RNA are essential for activity *in vivo*⁴³. The specificity of telomerase inactivation by oligonucleotide 3-directed cutting within the CAACCCCAA sequence, compared with the cutting near this sequence directed by oligonucleotide 8, is evidence for the important nature of the CAACCCCAA sequence in telomerase activity.

Discussion

CAACCCCAA sequence may provide a template for TTGGGG repeat synthesis. We have identified and cloned the gene for an

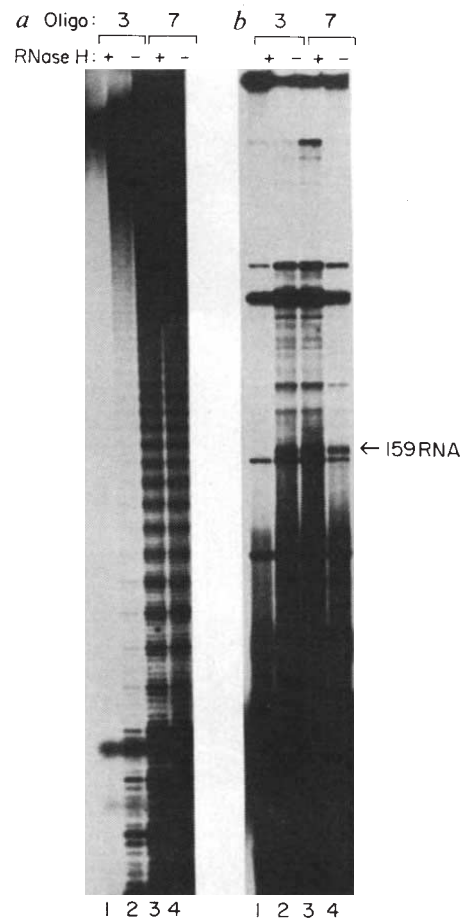


Fig. 4 *a*, The activity of telomerase which had been pretreated with various oligonucleotides in the presence or absence of RNase H and then pelleted in a ultracentrifuge to remove excess oligonucleotide. RNase H was added during the preincubation to the reactions shown in lanes 1 and 3, but not in lanes 2 and 4. The oligonucleotides used in the preincubation are indicated above the lanes. The low level of activity seen in lane 2 relative to lanes 3 and 4 is due to the inhibitory effects of residual oligonucleotide 3 in these fractions. The dark material seen at the top of the gel in lane 1 is due to labelling of high relative molecular mass nucleic acid by some enzyme in the partial purified fraction. More highly purified telomerase fractions do not show this label accumulation. *b*, RNA from the pellets in *a* was extracted, 3' end-labelled with [³²P] pCp and run on a sequencing gel. The lanes are as in *a*. The position of the 159-base telomerase RNA is indicated with an arrow.

Methods. 200 µl of telomerase purified as described in the Fig. 2 legend was preincubated with 6 µl of the appropriate oligonucleotide at 1 µg µl⁻¹ in the presence or absence of 5 µl of RNase H at 2U µl⁻¹. Preincubation was at room temperature for 15 min. The extracts were then transferred to Beckman microfuge tubes and spun at 75,000 r.p.m. in a Beckman TLA-100.3 rotor at 4 °C for 3 hours. The supernatant was removed carefully and the pellet was resuspended in 100 µl of TMG+0.1 M NaCl. 20 µl was immediately assayed in the standard activity assay (see Fig. 2 and ref. 5). To the remaining 80 µl, 10 µg of proteinase K was added and the mix was incubated at room temperature for 15 min. The preparation was then phenol-extracted, ethanol-precipitated, 3' end-labelled with pCp as described⁵ and run out on a 6% polyacrylamide/7 M urea sequencing gel.

essential RNA component of telomerase. Several lines of evidence indicate that the synthesis of TTGGGG repeats is directed by an RNA template. First, the only RNA which reproducibly co-purified over numerous columns contains the sequence CAACCCCAA. Second, oligonucleotide 8 whose 3' end base pairs just downstream from the CAACCCCAA sequence is efficiently elongated by telomerase, whereas all other

non-telomeric sequences are not. Third, oligonucleotide 3, which is complementary to the telomerase RNA sequences up to and including the CAACCCCAA sequence, inhibits telomerase activity. Finally, cleavage by oligonucleotide 3 and RNase H within the CAACCCCAA sequence specifically inactivates telomerase activity. These observations suggest that the CAACCCCAA sequence of the telomerase RNA is in the active site of the RNP and that this sequence provides a template for synthesis of TTGGGG repeats.

Primer elongation by telomerase can result from two modes of primer recognition. Preincubation of telomerase with d(TTGGGG)₄ and RNase H did not inhibit enzyme activity (Fig. 2) or result in cleavage of the telomerase RNA, whereas there was RNA cleavage when d(TTGGGG)₄ was incubated with naked telomerase RNA and RNase H (data not shown). In contrast, base pairing of both oligonucleotide 3 and oligonucleotide 8 with the telomerase RNA in the intact RNP can direct cleavage by RNase H. Cleavage by RNase H can occur with as few as six base pairs of DNA-RNA duplex⁴⁴. Thus, the base-pairing interaction of oligonucleotides 3 and 8 with the telomerase RNP must be distinct from the way in which d(TTGGGG)₄ interacts with the RNP during the preincubation. This difference could be due to an alteration in the RNP structure induced by oligonucleotides 3 and 8, or it may reflect a real distinction between telomerase recognition and elongation. The initial recognition of telomeric primers may not involve the CAACCCCAA sequence of the RNA. This is consistent with our earlier observations that G-rich oligonucleotides with differing primary sequences corresponding to the telomeric sequence of a number of species, as well as synthetic telomere-like sequences, all efficiently prime repeat addition by telomerase^{5,45}. Together, these data indicate that a primer can be delivered to the active site in two ways; telomeric G-rich sequence oligonucleotides are specifically recognized, possibly by their unusual secondary structural features⁴⁶, whereas some oligonucleotides complementary to the 159 RNA (like 3 and 8) can be positioned in the active site by base hybridization.

The elongation-translocation mechanism. When oligonucleotide 8 was preincubated with telomerase and d(TTGGGG)₄ was added at time zero, the oligonucleotide 8-primed pattern was seen in the products, indicating that the elongation reaction is processive. This, together with the evidence that hundreds of repeats of TTGGGG are synthesized on the input primer, and that there are one and a half repeats of the template sequence, indicate that an elongation-translocation mechanism operates to synthesize many tandem repeats. Previous work showed that the sequence at the 3' end of a telomeric primer is recognized by telomerase in the elongation reaction. A primer ending in TTGGGG 3' is distinguished from those ending in TTGGG 3', GGGGTT 3' or TTGG 3'^{5,45} and each primer is correctly filled out to complete the last TTGGGG sequence before further repeats are added. These data could be explained by the mechanism outlined in Fig. 6. After primer recognition, the most 3' nucleotides of the primer are hybridized to the 3' portion of the CAACCCCAA sequence and the primer is filled out to complete the last full hexanucleotide repeat. Translocation then repositions the primer so that its most 3' nucleotides are again hybridized ready for elongation.

Such an elongation-translocation mechanism could contribute to the strong six-base periodicity of the *in vitro* addition reaction. Earlier work showed that varying the relative concentrations of dTTP and dGTP in the reaction could change the intensity of the bands in the *in vitro* synthesized banding pattern⁴. This dependence on nucleotide precursor concentrations is also seen for the *Oxytricha* and *Euplotes* enzymes (ref. 23 and D. Shippen-Lentz and E. Blackburn, submitted). Pausing upon the addition of dTTP, as well as primer translocation, may contribute to the six base periodicity in the *in vitro* reaction products. Depending on the nucleotide concentration used, either two or three strong stops are seen in the elongation pattern using (TTGGGG)₄ as

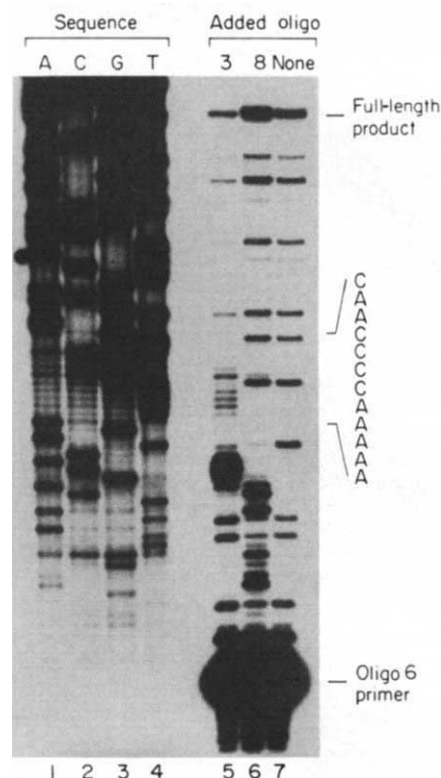


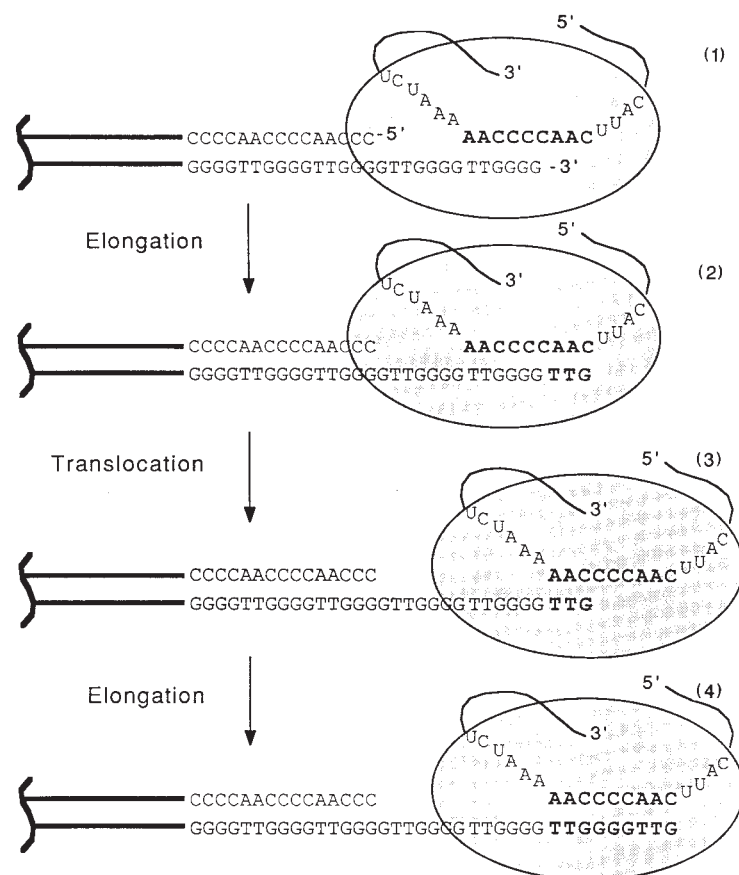
Fig. 5 Primer extension analysis of telomerase RNA cut with RNase H. Lanes 1-4, the sequencing ladder marker using oligonucleotide 6 as a primer. Lanes 5-7, the extension products from [³²P] oligonucleotide 6 on RNA extracted from telomerase fractions after treatment with RNase H and the oligonucleotides indicated above the lanes. The bands which appear in all of the lanes including lane 7, where no oligo was added, were probably due to the secondary structure of the 159 RNA. The full-length primer extension product is seen in all of the lanes because the cutting by RNase H in this experiment was not complete. The region corresponding to the CAACCCCAA sequence is indicated.

Methods. 400 µl of telomerase purified over a sizing column and heparin agarose, were incubated with 10 µl of the indicated oligonucleotide and 10 µl of RNase H (2U µl⁻¹) at room temperature for 30 min. 40 µl of a 10% SDS + 100 mM EDTA solution and 10 µg of proteinase K were added. The mix was incubated at room temperature for 20 min and then the RNA was phenol extracted and ethanol precipitated after the addition of 5 µg of carrier tRNA. The pellet was resuspended in 6 µl of 125 mM KCl and 5 mM Tris pH 8.3. To 3 µl of RNA, 1 µl of [³²P] oligo 6 containing 0.05 µg was added and each mix was incubated at 65 °C for 5 min. The annealing reactions were then transferred to 42 °C for 1 hour. To each reaction 6 µl of elongation mix was added. The elongation mix contained 2 µl 5x reverse transcriptase buffer, 1 µl each dNTP at 2.5 mM, 1 µl avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences), 2 µl DEP-water. The elongation was carried out at 52 °C for 30 min. Sequencing loading dye (4 µl) was then added and 4 µl of each sample was loaded onto a 6% polyacrylamide sequencing gel. The sequencing markers were produced using oligonucleotide 6 as a primer following the procedure for supercoiled DNA sequencing using the Sequenase kit (USB).

the primer. The two strong stops correspond to the two T residues in the repeat⁴ whereas the three stops correspond to the addition of the nucleotides TTG (data not shown). This pausing pattern is consistent with the model presented in Fig. 6 for elongation and translocation.

Implications of the internal RNA template. The specification of TTGGGG repeat addition by an internal template is analogous to the utilization of the internal binding site as a template in the reactions catalysed by the *Tetrahymena* intron ribozyme. This ribozyme has been shown to catalyse self splicing, trans-splicing, RNA endonuclease and RNA polymerase reactions⁴⁷⁻⁵². In all of these reactions a sequence internal to the

Fig. 6 Model for elongation of telomeres by telomerase. The *Tetrahymena* telomere is shown containing a 13-base overhanging TTGGGG strand⁵⁴. (1) After recognition of the TTGGGG strand by telomerase, the 3' most nucleotides are hybridized to the CAACCCAA sequence in the RNA. (2) The sequence TTG is then added one nucleotide at a time⁴. (3) Translocation then repositions the 3' end of the TTGGGG strand such that the 3' most TTG nucleotides are hybridized to the RNA component of telomerase. (4) Elongation occurs again, copying the template sequence to complete the TTGGGGTTG sequence. This mechanism explains how oligonucleotides with 3' ends terminating at any nucleotide within the sequence TTGGGG are correctly elongated to yield perfect tandem repeats of (TTGGGG)_n (ref. 5).



ribozyme interacts with a substrate through Watson-Crick base pairing. Mutations in this internal binding site alter the substrate specificity of the ribozyme⁴⁷. By analogy, it is predicted that changing the sequence of the internal template of telomerase would result in synthesis of telomeric repeats of that altered sequence. Whether the analogy between the telomerase and the intron ribozyme of *Tetrahymena* extends to include catalysis by RNA alone is a question which we can now begin to address.

DNA replication and RNA transcription represent conventional nucleic acid synthesis reactions directed by an external template. Terminal deoxynucleotidyl transferase, poly(A) polymerase and tRNA nucleotidyl transferase represent completely template-independent mechanisms of nucleic acid synthesis. In contrast to both of these types of polymerases, the *Tetrahymena* intron ribozyme and telomerase represent a new class of polymerase which is characterized by containing a template sequence that is an integral component of the polymerase itself. Although

many RNPs, as well as the *Tetrahymena* ribozyme, use base pairing of their RNA components with other RNAs to determine reaction specificity^{24-28,53}, the telomerase enzyme is unique among eukaryotic RNPs in that it uses an RNA template for DNA synthesis.

The synthesis of DNA using an RNA template may be a very ancient process. Telomerase enzymes with RNA components may be relics that have descended from an ancestral type of DNA polymerase whose action was based on an internal RNA template. In this model, the primitive DNA polymerase was replaced in evolution by the current protein-based DNA polymerases and reverse transcriptases which use exogenous templates, whereas telomerase retained the endogenous template and was used by eukaryotes for telomere maintenance.

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LETTERS TO NATURE

Is N44C a fossil X-ray ionized nebula?

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Long-slit spectroscopic CCD observations of the H II region N44C (refs 1–3) in the Large Magellanic Cloud and its central hot, massive star^{2,3} show that the stellar temperature is far too low to account for the high excitation of the nebula. In particular the strong nebular He II 4686-Å recombination radiation, which is absent in normal H II regions, cannot be understood in terms of ionization by the stellar ultraviolet flux. Here we propose an alternative interpretation in terms of a (fossil) X-ray photo-ionized nebula resembling the recently discovered He III region around the luminous X-ray binary LMC X-1 (ref. 4). In this picture the presently extinct X-ray source that has been responsible for the high ionization in N44C could well be identical with the transient LMC X-5 (refs 5, 6). From photon-counting arguments and recombination timescales of the interstellar plasma we find that less than 100 years ago the X-ray luminosity should have been comparable with that of the most luminous X-ray binaries (a few $\times 10^{38}$ erg s⁻¹). This hypothesis can be tested by future observations.

Among the most conspicuous differences that distinguish emission line spectra of H II regions from those of the generally more highly photo-ionized planetary nebulae is the absence, in the former, of the He II 4,686-Å recombination line, because even the hottest known massive stars (O stars) radiate only negligible amounts of photons more energetic than 4 Rydbergs (54.4 eV), which are of sufficient energy to ionize helium completely^{7,8} (see below). A notable exception is the nebula N44C (NGC1936) in the Large Magellanic Cloud (LMC), which was reported¹ to exhibit an unusually high-excitation planetary-nebula-type spectrum that includes strong He II 4,686-Å recombination radiation ($I_{4,686}/I_{H\beta} = 0.07$). Subsequently², this region was studied by narrow-band photographic imaging of H α and [O III] 5,007-Å emission surrounding a central star, which was proposed as the cause of the ionization.

These investigations have not been followed up until recently, when the nebular He II 4,686-Å emission was rediscovered³ and an effective stellar temperature of 70,000 K was derived from the nebular recombination-line flux and the optical brightness ($V = 14.6$; K. Reinsch, personal communication) of the central star (star 2), using the Zanstra method. Luminous stars with such high temperatures have so far not been detected (the earliest spectral types, O3, correspond to $T_{\text{eff}} = 45,000$ –53,000 K)^{9,10}, but they are nevertheless predicted to exist during late (helium-burning) phases in the evolution of very massive stars^{11–14}. Because of strong mass loss in a stellar wind, however, the surfaces of stars initially more massive than $\sim 40 M_{\odot}$ are expected to become strongly depleted in hydrogen and should appear as hot Wolf–Rayet (WR) stars^{11–13}. Whereas the issue of WR-star temperatures is a matter of current debate (see ref. 15 and references therein), we note that some WR stars indeed emit a luminous He⁺ Lyman continuum capable of photo-ionizing large He III regions. Examples of such systems are the galactic nebula G2.4+1.4 around the WO1 star LSS4368 (ref. 32) and part of the nebula N76A in the Small Magellanic Cloud, which is ionized by the WN star AB7 (M.W.P. *et al.*, manuscript in preparation). Because of heavy contamination by the nebular

emission lines of N44C, the previously reported³ spectrum of star 2 does not provide good indications of photospheric temperature and hydrogen abundance; the lack of broad emission-line features, however, already excludes a WR-type nature. Here we report results that do not support the suggestion of a very high surface temperature, and we suggest an alternative explanation for the high degree of ionization in N44C in terms of a fossil X-ray ionized nebula.

During four consecutive nights in January 1987 we carried out long-slit spectroscopic observations of N44C and its central star using the B&C spectrograph on the ESO 3.6-m telescope with a 520 $\times$ 320-pixel (30 μ m size) RCA CCD detector and a spectral full-width-at-half-maximum (FWHM) resolution of 5.5 Å (1.6 pixel). Several different slit orientations were chosen to cover as much of the emission-line region as possible. The intensities of several diagnostic nebular lines were extracted as a function of position along the slit, and background stellar and nebular continuum emission at nearby wavelengths were subtracted (see ref. 4 for method). In these intensity histograms stellar absorption/emission lines appear as corresponding features with a width determined by the seeing profile during the observations (1.5–2.5 arcsec FWHM). Figure 1 illustrates the strong concentration of the H I and He I nebular intensity and the increase in helium and oxygen ionization towards the central star 2. The mean extent of the He III region, as measured from the He II 4,686-Å recombination line, amounts to 18 arcsec, corresponding to 4.5 pc at the distance of the LMC, but this emission can be traced even to 35 arcsec (9 pc) in a radial filament southwards of the centre. Unresolved dips are clearly present in all H-Balmer-line and He I 4,471-Å intensity histograms (corresponding to different slit orientations) at the position of star 2 (Fig. 1a and b). The latter feature is ~ 5 –10 times narrower than the broad 4,471-Å/H β minimum around star 2, reflecting the reduced He I emissivity (proportional to He⁺ density) in the He III region. We are confident, therefore, that the O star exhibits photospheric He I absorption with an estimated equivalent width (EW) of 0.2–0.4 Å.

From the CCD images we have also extracted the optical spectrum of star 2 by careful subtraction of the nebular contribution. Intensities and line ratios change so rapidly across N44C (and from pixel to pixel along the slit) that the elimination of nebular emission is far from perfect, causing spurious emission and/or absorption lines of nebular origin (Fig. 2). Nevertheless, the photospheric Balmer series in absorption confirms the presence of hydrogen in the atmosphere of star 2. Moreover, background subtracted spectra, aimed at an optimal cancellation of nebular Balmer lines, show that the H γ absorption EW (2.2 Å) is typical of early O-type stars^{9,10} and therefore imply normal hydrogen abundance. This result argues against star 2 representing an advanced hot stage of massive stellar evolution, because hydrogen would then be already strongly depleted on the surface^{11–13} unless a very metal-poor composition and small mass-loss rate are assumed¹⁴. From spectral classification and temperature calibration of O stars^{10,16}, the observed He I 4,471-Å/He II 4,540-Å EW ratio (0.2–0.5) implies a spectral type O4 to O6 and an effective temperature in the range of 40,000–50,000 K. That star 2 is probably a moderately early main-sequence O star with little indication of mass loss is also supported by the lack of the N V 4,604–4,620-Å, N IV 4,058-Å and N III 4,634–4,641-Å features that are seen in early Of stars¹⁶. The absence of stellar He II 4,686-Å emission indicates that the star is not a giant or supergiant.

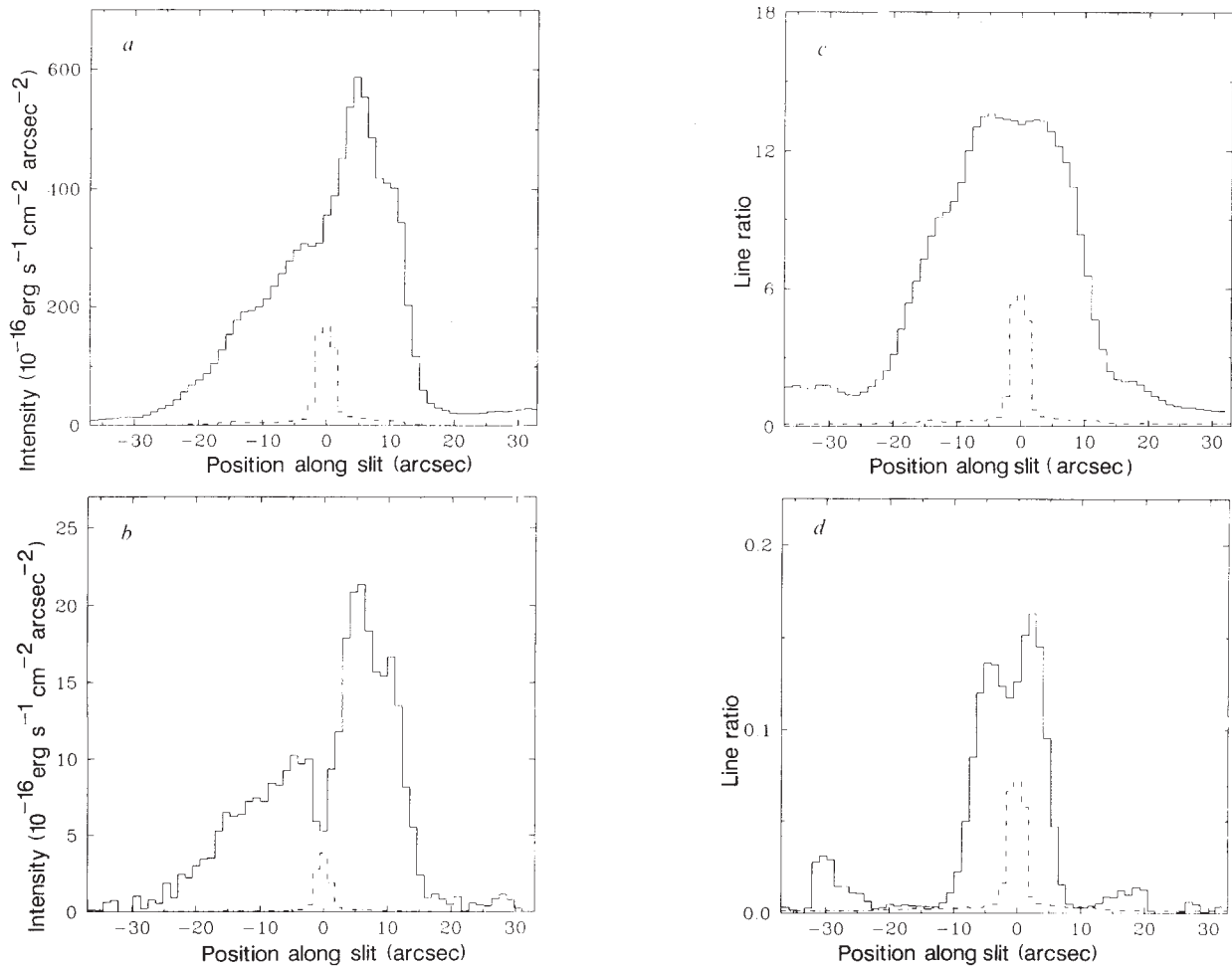


Fig. 1 Histograms of H β (a) and He I 4,471-Å (b) emission, and [O III] 4,959-5,007-Å/[O II] 3,727-Å (c) and He II 4,686-Å/H β (d) line ratios, in N44C as a function of position along the slit (position angle 313.°3; SE: left; NW: right). Stellar and nebular continuum flux at nearby wavelengths has been subtracted. The spatially unresolved dip in b indicates photospheric He I absorption in star 2; compare Fig. 2. The ionization structure in c and d is highly symmetric and is centred on star 2. The stellar continuum radiation scaled down by an arbitrary factor is shown for comparison in the dot-dashed curves. 1 arcsec corresponds to 0.25 pc in the LMC. The extent of the 4,686-Å emission (He III region) is 4.5 pc.

Recent calculations of nonlocal-thermodynamic-equilibrium-model atmospheres¹⁷ of O stars indicate that the He⁺ Lyman continuum can be strongly enhanced relative to plane-parallel models without stellar wind⁷, when spherical extension and mass loss are taken into account. We have computed the expected

4,686-Å recombination flux, $F_{4,686}$, from such a model for the O4f star ζ Pup, to which star 2 is comparable except for its much weaker stellar wind. Using the well-known Zanstra method to link $F_{4,686}$ to the stellar brightness in the visual, we find that this model underestimates the 4,686-Å flux, measured as $1 \times$

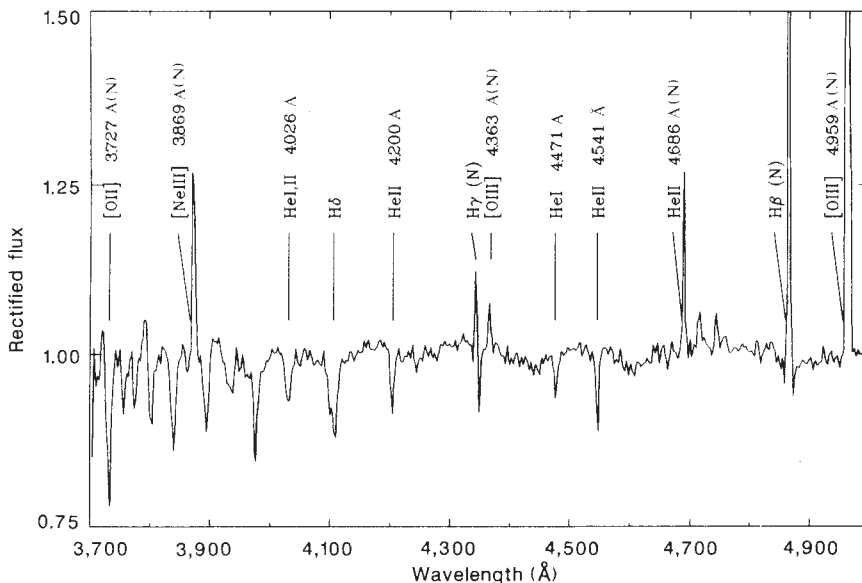


Fig. 2 Rectified optical spectrum of star 2. Spurious emission and absorption features (marked N) are due to imperfect subtraction of the nebular background. Note the presence of photospheric H I, He I and He II absorption lines, indicative of an O4-O6 V spectral type.

$10^{-12} \text{ erg cm}^{-2} \text{ s}^{-1}$ (note that the value reported in ref. 3 is in error (G. Stasinska, personal communication)), by a factor of 130. Our observations therefore do not support the suggestion that star 2 is an extremely hot star capable of producing the N44C ionization structure.

An alternative interpretation³, in terms of ionization by a strong stellar wind or supernova blast wave colliding with the interstellar medium, is also inconsistent with the observations. In this mechanism, high shock velocities in excess of 100 km s^{-1} , and accordingly high electron temperatures ($T_e(\text{O}^{2+}) > 24,000 \text{ K}$), in the O III region are required to produce substantial fractions of He^{2+} ions¹⁸, whereas the observed temperature³ (12,000 K) clearly indicates that N44C is photo-ionized. We also note that the radial velocity of the $4,686\text{-}\text{\AA}$ emission, $302 \pm 5 \text{ km s}^{-1}$, corresponds within 5 km s^{-1} to those of lower ionized species in the N44 complex, which effectively rules out a chance projection of a galactic planetary nebula onto the face of the LMC.

Guided by the strong resemblance of the optical emission-line spectrum to that of the He III region N159F surrounding the luminous X-ray binary LMC X-1 (ref. 4), we explored the conjecture that N44C represents an X-ray photo-ionized nebula (XIN). However, in the 0.15–4.5-keV Einstein satellite survey of the LMC (ref. 19), no X-ray emission from the vicinity of N44C was detected, and re-analysis of a previously unpublished Image Proportional Counter (IPC) observation of N44 (8,000 s integration time on 2 July 1980; F. Seward, personal communication) places an upper limit of $2 \times 10^{35} \text{ erg s}^{-1}$ on non-resolved X-ray emission from star 2. This value is 2,000 times smaller than the XIN hypothesis would predict (see below), excluding the possibility that the X-ray source was sufficiently active at that time. On the other hand, there is independent support for earlier X-ray activity in 1974–1976 from OSO-7 and Ariel V observations^{5,6}, which reveal a soft (2-keV) variable source, LMC X-5, at a celestial position consistent with that of N44C, although the relatively large errors preclude an unequivocal identification^{19,20}.

Adopting the fossil XIN interpretation of N44C, we can estimate the former luminosity of the currently inactive X-ray source. Drawing on photon-counting arguments⁴ for the XIN around LMC X-1 and assuming the same soft X-ray and extreme ultraviolet spectral distribution for both sources, the observed flux $F_{4,686}$ corresponds to a recombination line luminosity, $L_{4,686}$ of $3 \times 10^{35} \text{ erg s}^{-1}$, and therefore implies a 2–10-keV X-ray luminosity, L_x of $\sim (2\text{--}4) \times 10^{38} \text{ erg s}^{-1}$. As some $\text{He}^{2+} \rightarrow \text{He}^+$ recombination must have occurred since X-ray activity ceased, this value represents a lower limit, implying that the source must have ranked amongst the most luminous X-ray binaries known.

A further important indicator of high nebular ionization is $[\text{Ne v}] 3,426\text{-}\text{\AA}$ emission, which line is produced at an ionization potential of 97.1 eV. This line is present in the XIN N159F (ref. 4) with a maximum intensity relative to $\text{H}\beta$, $I_{3,426}/I_{\beta}$, of 0.3, but is not detected in N44C with an upper limit of 0.05. How can this observation be reconciled with the fossil XIN hypothesis? To answer this question we note that recombination of Ne^{4+} is determined mainly by electron capture; charge-transfer reactions with H^0 are negligible because the ultraviolet radiation of star 2 maintains strong hydrogen ionization in the nebula. The recombination timescale is therefore $\tau_{\text{Ne}^{4+}} = (\alpha(\text{Ne}^{4+}, T) n_e)^{-1}$ where $\alpha(\text{Ne}^{4+}, T) (=9 \times 10^{-12} \text{ cm}^3 \text{ s}^{-1}$ for $T = 10,000 \text{ K}$ (refs 21, 22)) is the Ne^{4+} recombination coefficient and n_e is the electron density. Therefore, $[\text{Ne v}] 3,426\text{-}\text{\AA}$ emission decays on a timescale of $16 (n_e/200 \text{ cm}^{-3})^{-1}$ years after the extinction of the X-ray source (an electron density of 200 cm^{-3} has been assumed to be representative for N44C (ref. 3)). As recombination of He^{2+} takes place on a longer timescale ($\tau_{\text{He}^{2+}} = [\alpha(\text{Ne}^{4+}, T)/\alpha(\text{He}^{2+}, T)] \tau_{\text{Ne}^{4+}} = 6.5 \tau_{\text{Ne}^{4+}}$), the combination of strong $4,686\text{-}\text{\AA}$ and the absence of $3,426\text{-}\text{\AA}$ radiation in N44C allows us to directly estimate the time, t_{off} , that has elapsed since the switch-off the X-ray source. Taking into

account light travel-time effects, we find $20 \text{ yr} < t_{\text{off}}(n_e/200 \text{ cm}^{-3}) < 100 \text{ yr}$. $\tau_{\text{He}^{2+}}$ also represents the timescale for the formation of the He III region²³, and therefore provides a lower limit, t_{on} , for the duration of strong X-ray activity in the past ($t_{\text{on}} > 10^2 (n_e/200 \text{ cm}^{-3})^{-1} \text{ yr}$).

The concept of fossil nebulae being in an ionization state that is inconsistent with the presently observed ultraviolet sources they contain has been discussed previously in the context of planetary nebulae²⁴, H II regions, active galaxies (ref. 25 and references therein) and the interstellar medium around young supernova remnants^{26,27}. However, the evolutionary timescales associated with significant decreases of the ionizing flux are in most cases much longer than the recombination and cooling timescales in the nebulae. The estimated lifetime of highly luminous massive X-ray binaries ($\sim 10^4\text{--}10^5 \text{ yr}$ (ref. 28)) seems also to be too large to render fossil XINs observable. On the other hand, several 'permanent' sources (as opposed to the short-lived transients) are known to switch off for extended ($> \text{several years}$) time intervals^{29,30}, demonstrating that in these systems, X-ray active phases can alternate with periods of quiescence on timescales much shorter than those determined by stellar evolution. (Although this phenomenon has not been observed previously in close X-ray binaries with O-star primaries.) This as yet poorly understood behaviour implies the existence of a non-negligible number of fossil XINs which are observable if the density of the interstellar medium is sufficiently high.

A key test of the XIN hypothesis for N44C is the prediction that orbital motion of the advocated close compact binary companion causes star 2 to exhibit periodic radial-velocity and possibly ellipsoidal light variations. From our observations we derive an upper limit of 60 km s^{-1} to any such velocity variations. This value is of the same order as the radial-velocity amplitudes displayed by the O-star companions of several luminous X-ray pulsars³¹ to which the proposed, presently inactive X-ray binary in N44C might be compared.

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Orbital evolution of circumplanetary dust by resonant charge variations

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Vast ethereal rings composed of micrometre-sized particles have been detected about the three giant planets so far visited by Voyager. Jupiter's diffuse halo, main ring¹ and gossamer ring² are visible because of the dust they contain. Small particles are also important constituents of Saturn's tenuous outlying rings: the contorted slender F ring³, the G ring⁴⁻⁶ and the extensive E ring^{6,7}. At Uranus, dust was discerned by imaging⁸ amidst, as well as inwards of, the nine previously known narrow rings; plasma measurements^{9,10} suggest that micrometre grains are also present in an orbit of 4.4 times the planet's radius, where Voyager 2 pierced the planet's equatorial plane. Here we identify an orbital evolution mechanism for gravitationally dominated dust that may temporarily overwhelm other processes, and we derive evolution rates that agree with numerical integrations; we also indicate briefly how this process could affect the location of the faint rings evident today.

The orbits of micrometre-sized grains in planetary rings are governed mainly by gravity, but electromagnetic forces provide significant perturbations^{11,12}. Such effects accumulate at discrete locations in the rings, Lorentz resonances¹³, where specific components of the perturbations match orbital periods and cause orbital eccentricity e and inclination i to be large. These positions may determine the boundaries of Jupiter's ring^{1,11,13}. But Lorentz resonances and electromagnetic processes in general have not previously been thought to change secularly the orbital size (semi-major axis a), although gyro-phase drift¹⁴ and diffusive transport¹⁵ may be effective for the tiniest grains (radius $s \leq 0.1 \mu\text{m}$)^{6, 12, 16, 17}. The orbital evolution of dust has been ascribed instead to plasma (Coulomb) drag, with the Poynting-Robertson effect being a lesser contributor^{15,16,18}.

Grains become electrically charged because any surface in space develops a potential through photo-ionization by solar radiation and through the currents carried by the local plasma to their surfaces. About the giant planets, photo-ionization can be ignored if the magnetospheric plasma is dense (number density $n \geq 10^2 \text{ cm}^{-3}$) and/or energetic. Thus, assuming perfect absorption of an isotropic and maxwellian plasma and no secondary emission, and ignoring the discrete nature of the charging process, the history of a particle's instantaneous charge Q is followed by summing the flows of positive and negative ions to the grain^{19,20}:

$$\frac{dQ}{dt} = \pi s^2 \eta q w \left[\left(1 + \frac{\alpha^2}{2w^2} + \frac{2q|\Phi|}{m_i w^2} \right) \text{erf} \left(\frac{w}{\alpha} \right) + \frac{\alpha}{w\sqrt{\pi}} e^{-(w/\alpha)^2} \right] - 2\sqrt{\frac{2\pi kT}{m_e}} s^2 \eta q e^{q\Phi/kT} \quad (1)$$

where q and m_e are the electron charge and mass, w is the speed of the grain relative to the mean plasma (assumed co-rotating with the planet), T is the plasma temperature, $\alpha = \sqrt{2kT/m_i}$ is the thermal speed of the ions, and k is the Boltzmann constant. The grain's potential $\Phi = Q/s$ reaches equilibrium when $dQ/dt = 0$. The equilibrium potential $\bar{\Phi}(T, w) = \bar{Q}/s$ is shown in Fig. 1 for plasma conditions that might apply to a jovian ring grain. The dependence of Φ on w principally reflects changes in the ion flux to the grain, because the electron flux is essentially

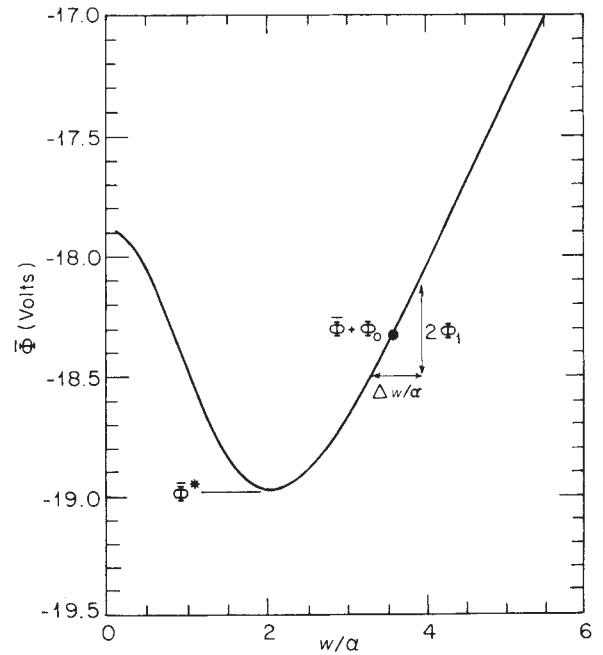


Fig. 1 A grain's equilibrium potential as a function of the ratio of w , the grain's speed through the plasma, to the ion thermal speed α for a plasma of O^+ at a temperature of 5 eV. Φ^* is the minimum potential that can be attained. Φ_1 is roughly the variation in the potential that occurs as w/α changes with high η .

independent of w when $\sqrt{2kT/m_e} \gg w$. A stationary grain develops a negative charge to ward off the swift electrons. For $w/\alpha \leq 2$, the ion current is reduced (because Coulomb attraction is less effective at focusing ions) so that $\bar{\Phi}$ decreases further, and eventually, at large w/α , the ion current is due primarily to ram charging onto the grain, so that $\bar{\Phi}$ increases linearly with w/α .

Q will vary systematically during the grain's orbit because the state of the plasma (η , T and even composition) varies with position and because a grain's velocity relative to the ambient co-rotating plasma varies along an elliptic orbit. Expanding equation (1) about $\bar{Q}$ (ref. 21) gives an inhomogeneous linear first-order differential equation for $\Delta Q = Q - \bar{Q}$ showing that small harmonic changes in w , η or T cause harmonic variations in ΔQ , which are phase-delayed because it takes some time to acquire the charge appropriate to local conditions. Allowing for large orbital excursions, the grain's charge can be expressed in general (compare refs 17 and 21) as

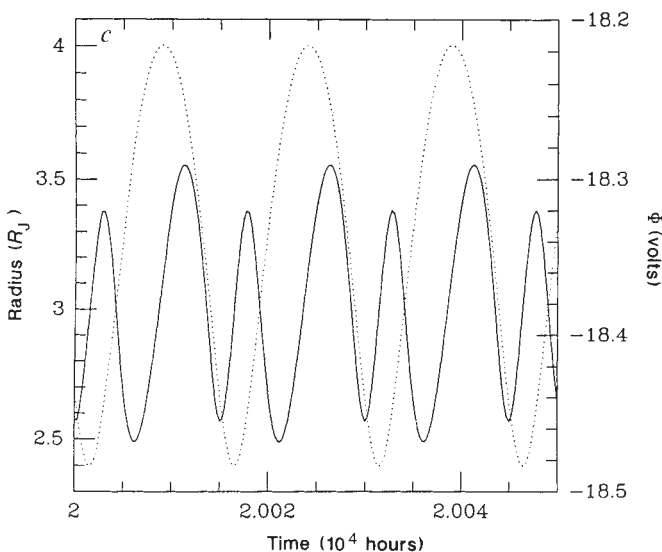
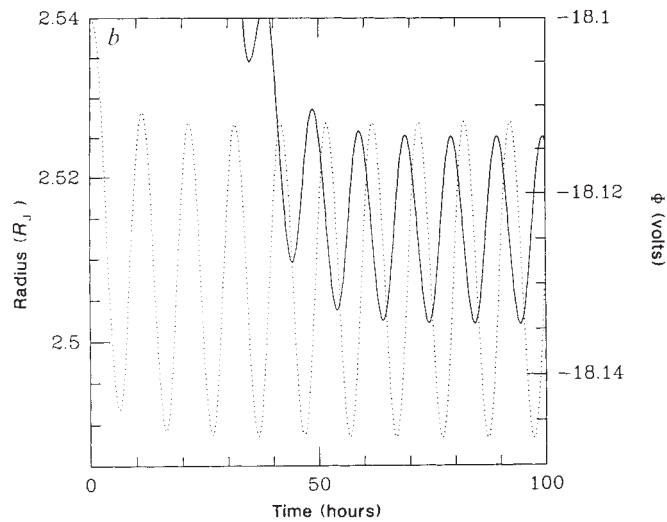
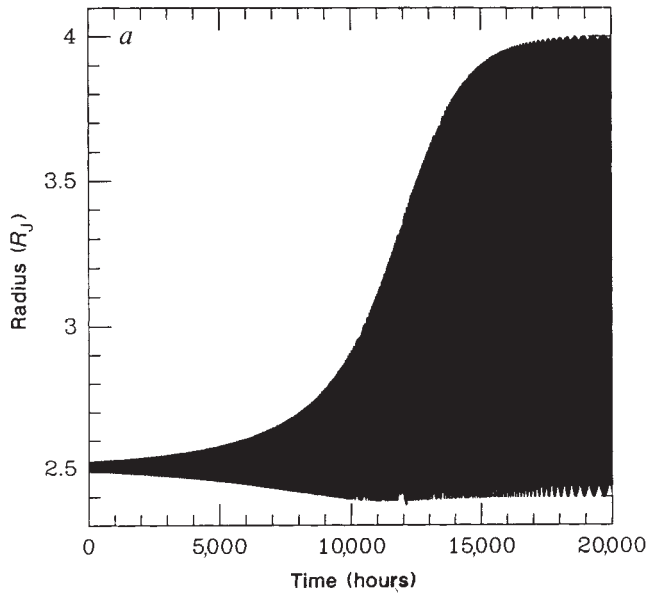
$$Q = \bar{Q} + Q_0 + Q_1 \cos(\theta - \theta_1) + Q_2 \cos 2(\theta - \theta_2) + \dots \quad (2)$$

where time has been replaced by θ , the orbit's true anomaly or its angular position from pericentre, and where (Q_i, θ_i) are the strengths and phase lags of the various Fourier terms, which will be functions of the mean orbital position.

Present evidence indicates that particle sizes and potentials in tenuous planetary rings are such that motions are gravitationally dominated^{6,11}. Here we concentrate on the orbital evolution caused by the charge variations produced, in turn, by velocity changes along an elliptical orbit. The case of electromagnetically dominated grains, where particles gyrate about and drift across magnetic field lines, has been analysed previously¹⁴ and applied to a jovian grain's evolution¹⁷.

Figure 1 indicates the charge variation along an orbit if the charging time is much less than an orbital period; $\bar{Q} + Q_0$ is then the mean charge for a particular orbit and Q_1 is roughly the maximum excursion about this value; Q_2 and higher-order

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terms are present only when large changes occur in w/α (so that linearization in velocity variation is inappropriate) or when the orbit is such that the minimum potential Φ^* (Fig. 1) is achieved. The Q_1 term indicates that a part of the charge varies with the orbital period; hence a delayed resonant perturbation acts on the particle, causing orbital evolution by a process that we call resonant charge variation.

We consider grains moving through the equatorial plane of an axially symmetric magnetic field:

$$\mathbf{B} = B_0(R/r)^3[1 + \beta(R/r)^3 + \dots]\hat{\phi}$$

where B_0 is the dipolar magnetic field at the planet's surface ($r = R$), $\beta (\ll 1)$ is the relative octopole strength and $\hat{\phi}$ is the unit normal to the equatorial plane in the direction of the planet's spin Ω . Near the planet, the plasma is carried by the magnetic field, producing, in the inertial reference frame, a 'co-rotational' electric field $\mathcal{E} = \Omega R b \hat{r}/c$, where c is the speed of light and $\hat{r}$ is the radial unit vector.

The orbital evolution of semi-major axis a and orbital eccentricity e is determined solely by the rates of change of the orbit's specific mechanical energy, $E = -GM/2a$, and angular momentum, $\mathbf{H} = \sqrt{GMa(1-e^2)}\hat{\phi}$ (ref. 22); G is Newton's gravitational constant and M is the planet's mass. Thus, $a^{-1} da/dt = \dot{E}/E$ and $de^2/dt = (e^2-1)(2\dot{H}/H + \dot{E}/E)$. To integrate $\dot{a}/a$ over an orbit in the inertial frame, only the work done against $\mathcal{E}$ needs to be considered because magnetic forces do no work ($\mathbf{v} \cdot (\mathbf{v} \times \mathbf{B}) = 0$). Thus the semi-major axis changes at the average rate

$$\frac{1}{a} \frac{\Delta a}{\Delta t} = \frac{2a}{mGP_0} \oint Q \mathcal{E} \cdot d\mathbf{r} \quad (3)$$

where m is the grain's mass and the path integral is taken over a complete orbital period P_0 . Using the magnetic-field expression above, we find

$$\frac{1}{a} \frac{\Delta a}{\Delta t} = \frac{B_0 \Omega (R/a)^3 e}{nmc(1-e^2)} \left\{ Q_1 \sin \theta_1 + \frac{\beta (R/a)^2}{(1-e^2)^2} \times [Q_1(1+e^2/4)\sin \theta_1 + Q_2 \sin 2\theta_2] + \dots \right\} \quad (4)$$

where $n = 2\pi/P_0 = (GM/a^3)^{1/2}$ is the orbital mean motion.

Similarly,

$$\Delta \mathbf{H} = \frac{B_0 R^3}{mcH} \oint Q \hat{r} d\theta \hat{\phi} \quad (5)$$

The co-rotational electric field does not enter because, being radial, it produces no torque. From this, the rate of change of e can be calculated; it is written most compactly as

$$\frac{\Delta e^2}{\Delta a/a} = (1-e^2) - (1-e^2)^{1/2} \left(\frac{n}{\Omega} \right) \quad (6)$$

which can also be derived from the problem's Jacobi integral²¹.

We can compare results from these analytical expressions with numerical simulations of a charged grain's evolution. We use a Runge-Kutta scheme^{11,21} that, given a grain's position and velocity, computes accelerations resulting from Lorentz

◀ Fig. 2 a, The orbital history of a 1-μm grain of density 1 g cm⁻³ placed in a 5-eV O⁺ plasma ($\eta = 1 \text{ cm}^{-3}$). The grain was launched on an initially circular equatorial orbit at 2.54 R_J , R_J being the radius of Jupiter. The parameters are chosen to produce rapid evolution. b, The grain's orbital radius r (dotted) and its electric potential Φ (solid) are shown with finer time resolution during the early evolutionary portion of Fig. 2a. The orbital period is roughly 10 hours. The phase lag θ_1 is evident. c, The same quantities as in b but plotted at a later time, when the charge variation is primarily doubly periodic and the orbit has stabilized. Because of the greater distance to Jupiter and the larger Lorentz force, the period has lengthened to almost 15 hours.

forces plus non-point-mass gravity and, through Newton's equations, the consequent orbital evolution; simultaneously, the grain's charge state is followed by using equation (1). Figure 2 shows the numerically determined orbital history of a jovian dust grain, using the appropriate values for Jupiter, $B_0 = -4.092$ G and $\beta = 0.028$ (compare ref. 11). The orbit quickly becomes eccentric because the initial path is not in centripetal equilibrium once the charge develops, which occurs rapidly. As deduced from Fig. 1 and equation (2), charge varies at first primarily with the orbital period, like w , and exhibits a phase lag θ_1 .

After $\sim 9,000$ hours, the grain evolves rapidly outward while its eccentricity grows. This drift can be understood from equations (3) or (4): because of the phase lag, the grain's mean charge is slightly more negative during its inbound motion than on its outbound path (see Fig. 3). The electric field thus does positive work during each radial oscillation and the orbit size expands. When e has reached a value of ~ 0.2 , the evolution slows dramatically, for two reasons. First, as Fig. 2c suggests and a Fourier transform attests, Q then varies primarily in the second harmonic, which, for the same charge variation, is 10^{-2} – 10^{-3} times as effective as Q_1 , according to equation (4). Q_2 has developed because, with such a large e , w/α ranges from about 0.2 to nearly 4 (see Fig. 1); in addition, θ becomes a multi-periodic function of time, inducing Q_2 . Second, despite the large excursion in w/α , charge variations (especially Q_1) are fairly small because the changes in w/α occur too swiftly for equation (1) to respond. The numerically computed rates, based on measured Q_i and θ_i , compare very favourably with our analytical expressions. We note that the very low η , chosen here to cause a large θ and a rapid evolution, is incompatible with our neglect of photo-ionization; under these circumstances, resonant pulsations in Q caused by passage through the shadow are important perturbations (M. Horanyi, personal communication).

In many of our numerical experiments the orbit size changes faster than would be expected from either plasma drag^{6,15,16} or Poynting-Robertson (P-R) drag²³. For example, during the steep-sloped mid-portion of Fig. 2a, $\dot{a}$ is about 250 cm s^{-1} , whereas the plasma drag and P-R evolution rates are two to three orders of magnitude less^{6,17}; in denser plasmas, Coulomb drag could, however, dominate orbital evolution because its strength would increase at the larger density whereas the evolution caused by resonant charge variation would decrease. Nevertheless, the orbit's stabilization, which is apparent in Fig. 2a and which, as we have argued, is implicit in the charging scheme when velocity variations drive equation (1), means that dust will not inevitably leave the system.

The orbital evolution (equations (4) and (6)) will depend upon the nature of the charge variation ($Q_1, Q_2, \dots, \theta_1, \theta_2, \dots$), which in turn will be fixed by the orbit, because the latter imposes the time history for w , T and η in the charging relation (equation (1)). Other orbits or different plasma environments can produce

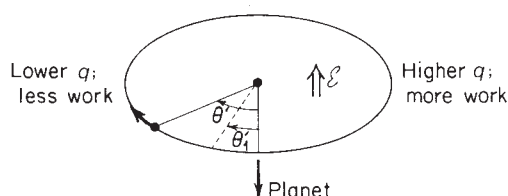


Fig. 3 The motion of a charged, orbiting grain as seen in a reference frame rotating at the mean orbital rate. The particle moves clockwise along this curve from pericentre (nearest the planet) to apocentre and back to pericentre. Because of the phase lag θ_1 , the mean charge on the right-hand side of the path is more negative than on the left-hand side so that more work is done moving toward the planet than away from it. A similar argument shows that the second harmonic is much less efficient in moving grains (compare equation (4)). For small eccentricity the plotted angle θ' approximately equals the true anomaly θ .

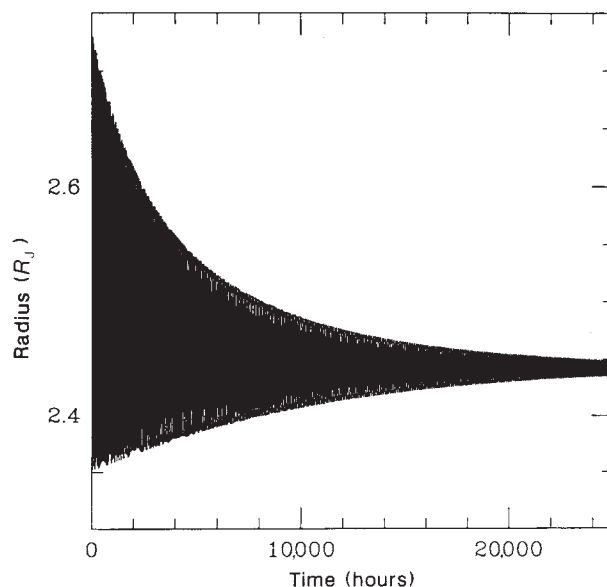


Fig. 4 The orbital history of a $0.34\text{-}\mu\text{m}$ grain of density 1 g cm^{-3} placed in a 0.2-eV O^+ plasma with density $\eta = 10 \text{ cm}^{-3}$. The particle is introduced with some initial radial velocity to provide enough eccentricity to initiate noticeable evolution.

dramatically distinct histories. Figure 4 shows a grain placed in the same position as for Fig. 2 but in a plasma such that the particle's potential is to the right of Φ^* on Fig. 1, and changes more quickly. A smaller grain size is chosen to make the perturbation forces comparable in the two examples. The grain's evolution is opposite, and the particle moves slowly toward the synchronous position while the orbit circularizes.

What might orbital evolution by resonant charge variation imply for the genesis of the faint rings? Because dust from a single source can be disseminated widely to populate a region both inside and outside the source (compare Fig. 2), it is possible for Enceladus to supply the whole E ring of Saturn, which straddles the satellite's orbit and extends out as far as Rhea's orbit. Similarly, the faint material surrounding Saturn's F ring^{6,24} may be the evolved debris from collisions occurring in the ring itself. Given appropriate plasma conditions in the jovian magnetosphere, grains from distributed parents in the gossamer ring could move toward synchronous orbit to produce the enhancement observed there (Fig. 4; compare refs 2 and 17); this would require either that w/α be more than several (which is difficult to achieve near synchronous orbit) or that T increase toward synchronous orbit. For Uranus, the dust detected at $4.4 R_U$ may have drifted inward from the outlying classical satellites rather than, as previously maintained¹⁰, necessarily being derived from the small moon 1985U1, the only known object between synchronous orbit and the point of detection.

Resonant charge variations can produce rapid orbital evolution of dust, but the precise rate, and even the direction, of evolution depends sensitively on plasma parameters and dust sizes in inner planetary magnetospheres. Because these quantities are not as yet well determined, it is very difficult to predict the transport of dust within faint planetary rings.

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The winter solstice phenomenon at Newgrange, Ireland: accident or design?

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On Midwinter's Day, around four and a half minutes after sunrise, the sun shines down the 'roof-box' of the Neolithic passage graves at Newgrange, Ireland, and illuminates the floor of its main chamber 18 m away. When the monument was constructed, however, first light would have occurred at sunrise in the form of a very narrow beam bisecting the chamber. Here I suggest that the width and height of the gap in the floor of the roof-box may have been deliberate, tracing the path of the Sun at the solstice. Newgrange predates the astronomical structures of Stonehenge by 1,000 years and as such may be the oldest astronomically orientated structure in the world.

Located some 50 km from Dublin, Newgrange is the largest in a group of Neolithic passage graves in the Boyne Valley, County Meath. Although discovered in 1699, the tomb was not properly excavated until the 1960s¹. It consists of a passage and chamber off which are three smaller alcoves (Fig. 1), giving the structure an overall cruciform appearance. Surrounding it is a large circular mound, over 80 m in diameter, of loose stones,

and encircling this again is an incomplete ring of standing stones. The mound and ring are, however, not concentric and probably not contemporaneous.

As early as 1909, Lockyer² remarked that the passage grave was approximately aligned to the rising Sun at midwinter. He did not, however, pay much attention to the site. During recent excavations of Newgrange, O'Kelly¹ unearthed a curious decorated structure located above the entrance to the tomb. This structure, which was later to be called the 'roof-box'¹, covered a vertical gap between the first and second roof slabs of the passage. Conscious of a local tradition that the chamber was illuminated by the Sun at a certain time of year, O'Kelly¹ investigated whether the roof-box had some solar function, and found that the midwinter Sun shone through the roof-box and gap to illuminate the central chamber of the tomb (Fig. 2) 18 m away. This event would have occurred even if access to the tomb was blocked by the entrance stone¹.

Patrick³ later concluded that the orientation of the roof-box towards the winter solstice was deliberate. Heggie⁴ has pointed out, however, that on the basis of Patrick's calculations, the Sun would illuminate the main chamber if its declination lay at any position between $-22^{\circ}58'$ and $-25^{\circ}53'$, and hence the probability of a solstitial alignment in the corresponding range of azimuths is about 1 in 13. Heggie⁴ concluded that this fact was 'not really significant enough to excite much interest'. But Heggie's calculation is in error for two reasons. First, as will be discussed below, Patrick's upper azimuth limit is about 1° too high and hence the declination range should be reduced accordingly. Second, and more importantly, the three-dimensional aspect of this problem has been ignored. As can be seen from Fig. 2, the solstitial light from the roof-box meets the floor at a glancing angle within the main chamber. If the gap between the first two roof-slabs was, say, 20 cm lower, or equivalently the passage was a few metres longer, sunlight would not enter the chamber. In fact, one would then observe the Boyne Valley, rather than the local horizon, through the gap. Correspondingly, if the gap was higher, the solstitial light would have been projected onto the back wall. Although such an alignment is of course admissible for statistical purposes, it would not have as dramatic an effect as that of a glancing beam on the floor. Thus the chance of Newgrange being accidentally aligned with a solstitial sunrise/sunset point is smaller, at least by a factor of two, than the figure quoted by Heggie⁴. Nevertheless the evidence still remains weak and it would not rival that found for sites such as Stonehenge⁴ and Kintraw⁵. To test the solar hypothesis further, we re-surveyed the roof-box, as seen from the main chamber, and the local horizon in the direction of sunrise on Midwinter's Day.

The passage itself is sinuous (Fig. 1), restricting the range of azimuths from which a ray of light can enter the main chamber. We found this range to be $133^{\circ}49'-137^{\circ}29'$ at the chamber

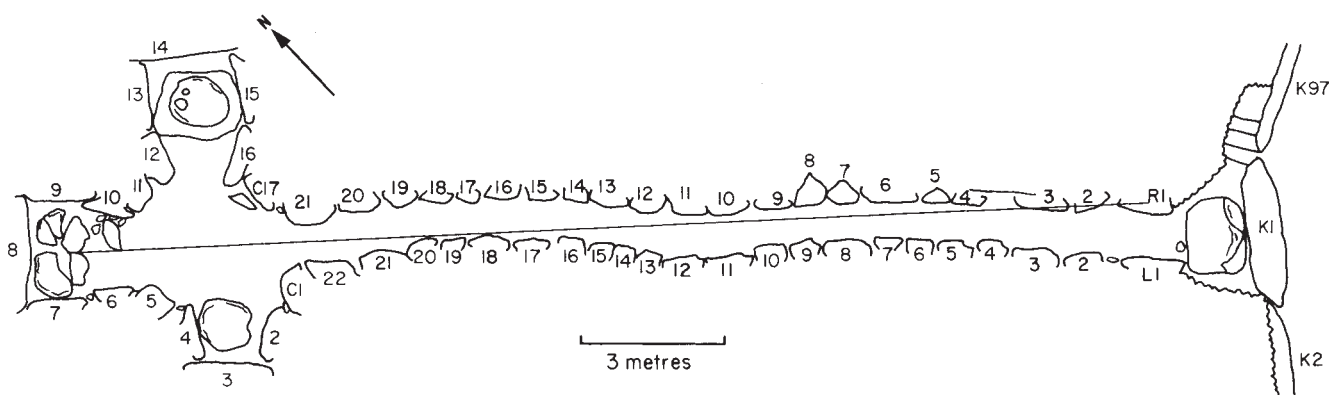


Fig. 1 Plan of Newgrange Tomb, displaying the passage, main chamber and its three alcoves. The marked line shows the minimum azimuth or 'first-light' axis of the passage. This axis coincides with the direction of Midwinter sunrise 5,150 years ago, when the tomb was built. Adapted with permission of C. O'Kelly.

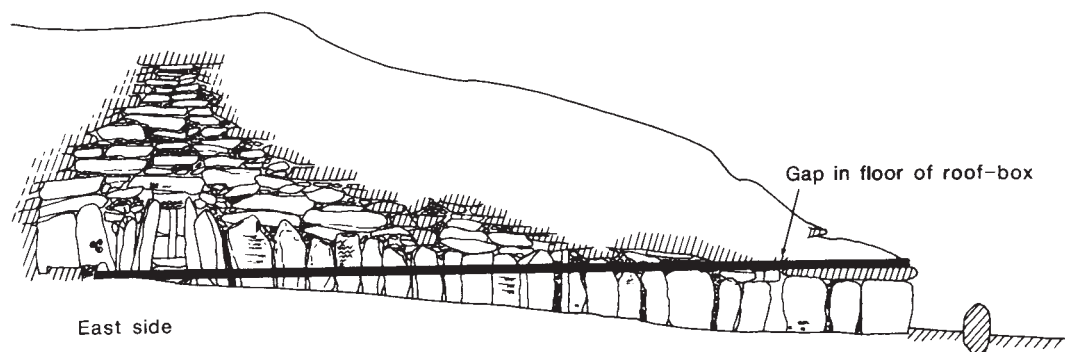


Fig. 2 Sectional elevation of the east side of Newgrange. The path of the first rays of the Sun around 3,150 BC is indicated.

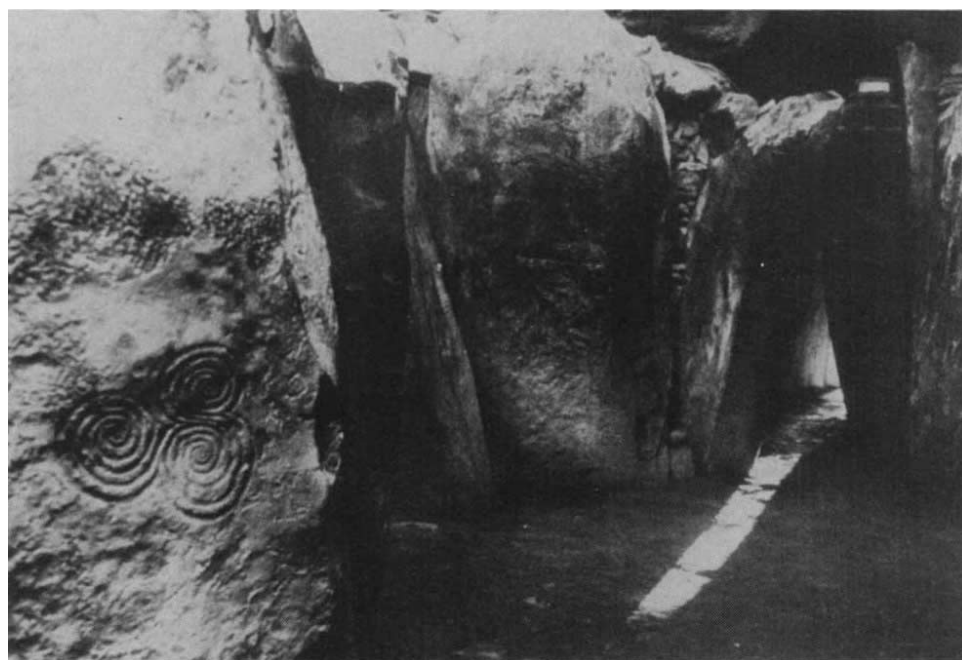


Fig. 3 Sunlight in the main chamber at the time of the winter solstice. The three-leafed spiral figure can be seen in the backmost alcove. Photo courtesy of Conn Brogan, Office of Public Works, Dublin.

entrance, with a typical error of about $\pm 3'$. The upper limit is $\sim 1^\circ$ greater than that quoted by Patrick³. We measured the light beam to be just under 34 cm wide near the entrance although it narrows towards the back recess, largely because stone L20 leans inwards. Close to the entrance to the chamber, however, L20 restricts the width of the light beam by 8 cm at most, so that if L20 were uprighted the minimum azimuth would be less than the quoted value by only $20'$. The chamber floor is slightly lower than the gap in the roof-box (Fig. 2) and hence at low altitudes, the light of the Sun is spread across the floor. The minimum altitude at which sunlight can enter the chamber is given by the height of the local horizon, $55' \pm 2'$, whereas the maximum altitude, confirmed by actual timing measurements of when the Sun disappeared (F. Prendergast, private communication) from the main chamber, is $2^\circ 11' \pm 2'$. Using height measurements of the gap behind the roof-box and the chamber floor, we found that light at minimum elevation would reach at least 1 m into the back recess (Fig. 1). However, sunlight is now seen to extend only as far as the edge of this alcove, because the minimum azimuth axis, or 'first light' axis, of the passage does not point towards midwinter sunrise. In fact, light from the right-hand limb of the Sun first enters the main chamber four and a half minutes after sunrise (F. Prendergast, personal communication), and at that time the Sun is almost completely above the horizon.

The question then arises as to what would have been the solar alignment when the monument was built. Carbon dating of two

charcoal samples from between the roof-slabs yielded radiocarbon ages of $4,450 \pm 40$ and $4,460 \pm 45$ BP¹. To convert this to a calendar date, we have used the dendrochronological record provided by samples of Irish Oak⁶. This indicates that the probable construction period for the site is $3,150 \pm 100$ BC. For these dates the obliquity of the ecliptic would have been $24^\circ 2' \pm 1'$ and the midwinter Sun would rise at an azimuth of $133^\circ 54' \pm 4'$. Here we have assumed, for calculating refraction⁸, a pressure of 1,010 mbar, a temperature of 0°C and the height of the local horizon to be $55' \pm 2'$. Variations in temperature and pressure make very little difference to the result. We see that the Sun would have risen just within the limits of the roof-box as seen from the main chamber. In other words, the 'first-light' axis of the passage corresponds, within $5'$ or so, to midwinter sunrise 5,150 years ago. If, at that time, one assumes that stone L20 was in an upright position, the accuracy would still be better than $25'$. It follows that when Newgrange was built, the first beam of sunlight on the floor was less than 10 cm wide, approximately 2 m long, and bisected the chamber (Fig. 1). Rather curiously, this beam would have entered the back recess and indirectly illuminated a three-leafed spiral figure on its wall (see Fig. 3), as mentioned in legend^{3,4,9}.

As the Sun's altitude and azimuth increased, the beam would have become gradually broader. The height of the gap behind the roof-box varies between 18 and 22 cm, being 20 cm on average. As seen from the main chamber 18 m away, this gap subtends an angle of roughly $38'$, so that the risen Sun is framed

in the vertical direction. Finally, the lower edge of the Sun would have left the roof-box, as seen from the main chamber, at an azimuth of $137^{\circ}42' \pm 5'$. Again this is very close to the maximum azimuth of the passage, implying a symmetry with respect to the Sun's passage at the winter solstice. Thus not only the height but also the width of the gap may be significant.

Newgrange predates the astronomically orientated structures (phase III) of Stonehenge by about 1,000 years. The evidence presented here supports the theory that the orientation of Newgrange was deliberate, which would make it therefore the oldest megalithic structure known for certain to have an astronomical function^{4,5}. The alignment at Newgrange was within a fraction (that is, within $\frac{1}{2}^{\circ}$) of the Sun's disk although it is doubtful whether the accuracy extends to the level of a few arcminutes. Newgrange should be seen in the light of large-scale studies such as those of Ruggles¹⁰, which show definitive evidence that megalithic man was interested in marking the southern limits in declination of the Sun and Moon, albeit

approximately. Such low accuracies suggest that ancient man's interest in these bodies may have been ritualistic rather than for the purpose of calendar construction.

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A superconducting copper oxide compound with electrons as the charge carriers

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Since the discovery of high-temperature superconductivity in copper oxide compounds¹, there has been much effort to understand what is required for a compound to have a high transition temperature (T_c). Up to now, all of the families of high- T_c copper oxide superconductors have contained two-dimensional sheets of Cu-O pyramids or octahedra, and the carriers of the superconducting current have been electron vacancies, or 'holes'. By contrast, we report here the discovery of a family of superconducting copper oxides in which the carriers are electrons. The new superconductors are Ce^{4+} -doped compounds, with the formula $Ln_{2-x}Ce_xCuO_{4-y}$, where Ln stands for the lanthanides Pr, Nd or Sm. The compounds have the Nd_2CuO_4 (T'-phase) structure², which is composed of sheets of Cu-O squares (see Fig. 1a). This structure has no apical oxygen atoms, in contrast to the T-phase structure with Cu-O octahedra (Fig. 1b), as observed in $La_{2-x}Sr_xCuO_4$ (refs 1, 2), and the T*-phase structure with Cu-O pyramids (Fig. 1c), as in $Nd_{2-x-z}Ce_xSr_zCuO_4$ (ref. 3 and Y. T. *et al.*, manuscript in preparation).

The superconducting materials were synthesized from a mixture of rare-earth metal oxides (CeO_2 , Pr_6O_{11} , Nd_2O_3 , Sm_2O_3) and CuO. The mixed powder was calcined in air at 950 °C for 10 hours, pressed into pellets and sintered in air at 1,150 °C for 12 hours. The samples were quenched in air to room temperature, and were found to be single-phase. To produce superconductivity, the Ce-doped samples were annealed for 10 hours under reducing conditions—at 1,000 °C in a stream of an Ar/O₂ gas mixture with $p_{O_2} < 10^{-3}$ atm—and quenched to room temperature in the same atmosphere. Best results were obtained with $p_{O_2} \approx 8 \times 10^{-5}$ atm.

Figure 1 shows the temperature dependence of resistivity for $Nd_{2-x}Ce_xCuO_{4-y}$. Although the undoped compound Nd_2CuO_4 is a typical semiconductor, doping with Ce^{4+} ions increases the conductivity (Y. T. *et al.*, manuscript in preparation). A remarkable decrease in resistivity was also observed in the undoped sample on quenching from 1,150 °C in air down to room temperature, which produces an oxygen deficiency ($y \approx 0.04$). These observations indicate that Ce^{4+} -doping and/or oxygen deficiencies introduce mobile electrons into the parent compound. The T'-phase Nd-based copper oxide cannot be doped with holes (Y. T. *et al.*, manuscript in preparation), whereas the T*-phase compound $Nd_{2-x-z}Ce_xSr_zCuO_4$ (Fig. 1c) can be doped with holes but not with electrons, which is also the case for the T-phase structure (Fig. 1b).

A fairly low resistivity ($\leq 10^{-2} \Omega \text{ cm}$) was observed for $Nd_{2-x}Ce_xCuO_{4-y}$ for $x=0.15$, the sample showing semi-metallic but not superconducting behaviour. (In Fig. 2, however, this sample shows a resistivity drop at around 9 K, which indi-

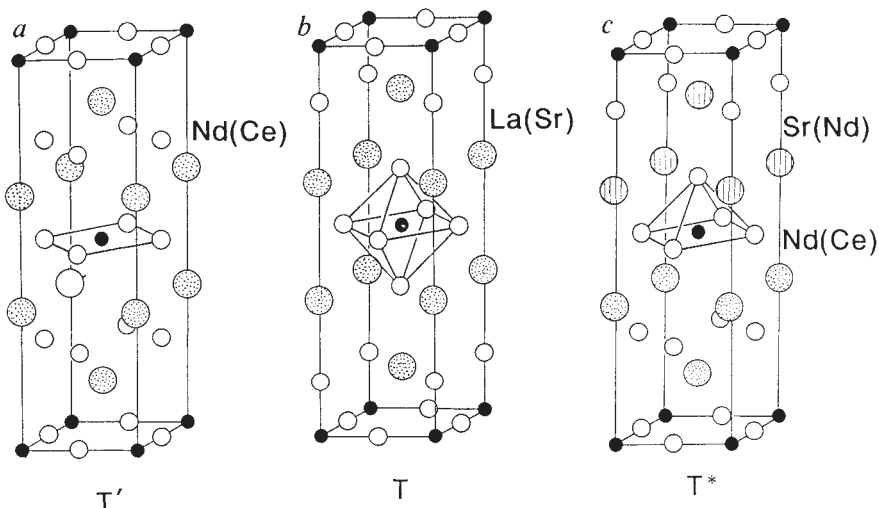


Fig. 1 Crystal structures of a, $Nd_{2-x}Ce_xCuO_4$ (T'-phase), b, $La_{2-x}Sr_xCuO_4$ (T-phase) and c, $Nd_{2-x-z}Ce_xSr_zCuO_4$ (T*-phase).

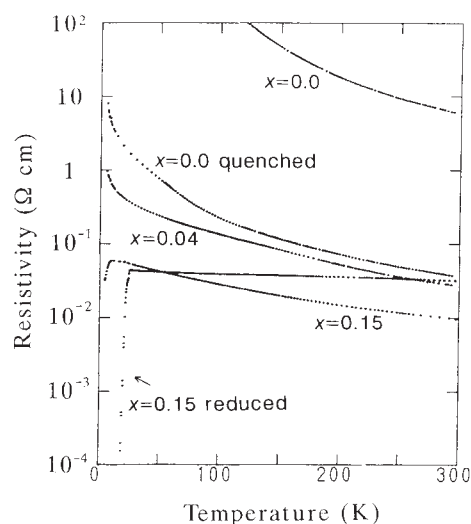


Fig. 2 Resistivity of $\text{Nd}_{2-x}\text{Ce}_x\text{CuO}_{4-y}$ as a function of temperature. In the $x=0.15$ sample, after reduction, there is an onset of superconductivity at $T_c \approx 24$ K.

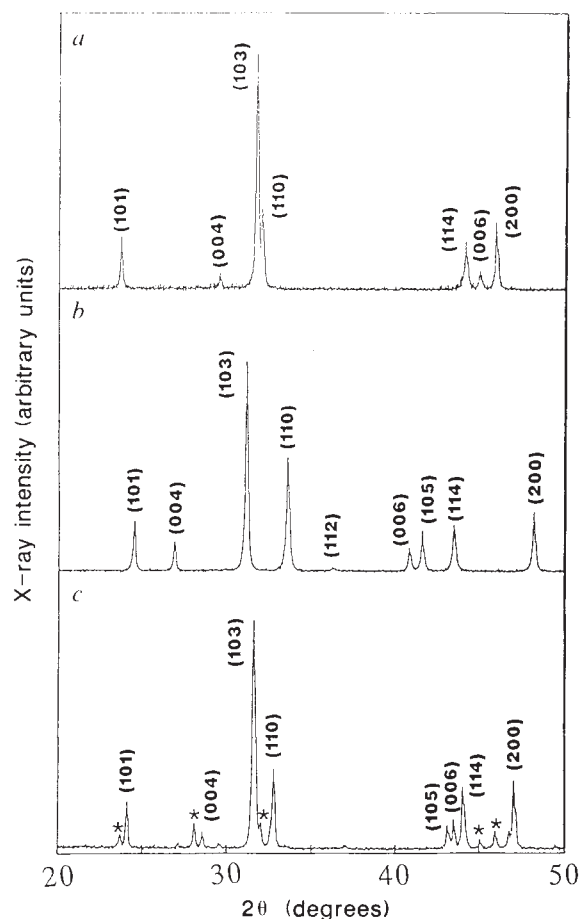


Fig. 3 Powder X-ray diffraction patterns of *a*, $\text{Nd}_{1.85}\text{Ce}_{0.15}\text{CuO}_{4-y}$ (T'-phase), *b*, $\text{La}_{1.85}\text{Sr}_{0.15}\text{CuO}_4$ (T-phase) and *c*, $\text{Nd}_{1.4}\text{Ce}_{0.2}\text{Sr}_{0.4}\text{CuO}_{4-y}$ (T*-phase). Asterisks indicate peaks from a secondary phase.

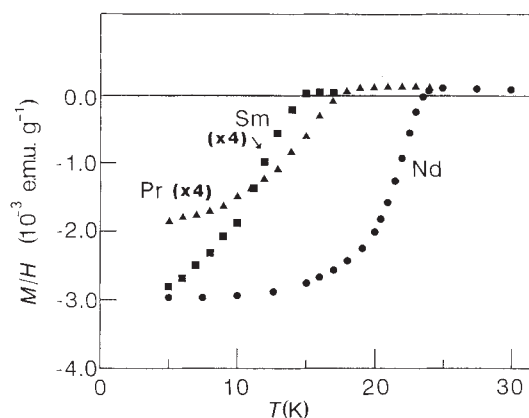


Fig. 4 Meissner signals in T'-phase $\text{Ln}_{2-x}\text{Ce}_x\text{CuO}_{4-y}$ (where Ln represents Pr, Nd or Sm). We plot the ratio of magnetization M to applied field H , for $H=10$ Oe.

cates superconductivity in a small portion of the sample.) Bulk superconductivity in the $x=0.15$ sample was achieved by the annealing procedure described above. Both resistivity and magnetization measurements indicated an onset of superconductivity at ~ 24 K. The concentration (q) of electrons introduced per [Cu-O] unit, or the effective copper valence ($2-q$), was estimated by an iodometric titration technique⁴ in which Ce^{4+} is reduced to Ce^{3+} . The $x=0.15$ sample used to obtain Fig. 2 has $q=0.20$ before the reducing procedure, and $q=0.28$ after this procedure (that is, when it is superconducting). This corresponds to an increase in oxygen vacancies ($y \approx 0.07$). Therefore, an increase in electron density beyond a certain value ($q \approx 0.20$) seems to initiate superconductivity in which electrons are the charge carriers.

That the carriers are electrons was also confirmed by measurements of the Hall coefficient. The sample $\text{Nd}_{1.85}\text{Ce}_{0.15}\text{CuO}_{3.93}$, with $T_c=24$ K, shows a negative Hall coefficient: $R_H = -6.5 \times 10^{-4} \text{ cm}^3 \text{ C}^{-1}$ at 300 K and $-2.3 \times 10^{-3} \text{ cm}^3 \text{ C}^{-1}$ at 80 K. This observation contrasts sharply with the other copper oxide superconductors so far known, in which positive Hall coefficients are always observed, indicating hole-type carriers.

The X-ray diffraction pattern for $\text{Nd}_{1.85}\text{Ce}_{0.15}\text{CuO}_{3.93}$ is shown in Fig. 3*a*, and, for comparison, those of the hole-superconducting T-phase $\text{La}_{1.85}\text{Sr}_{0.15}\text{CuO}_4$ (Fig. 3*b*) and T*-phase $\text{Nd}_{1.4}\text{Ce}_{0.2}\text{Sr}_{0.4}\text{CuO}_4$ (Fig. 3*c*) (see also Fig. 1). The diffraction pattern of $\text{Nd}_{1.85}\text{Ce}_{0.15}\text{CuO}_{3.93}$ is essentially identical to that of the undoped T'-phase compound Nd_2CuO_4 , and all diffraction peaks discernible in Fig. 3*a* can be indexed assuming the lattice constants of the typical tetragonal T'-phase structure², $a=3.95$ Å and $c=12.07$ Å. The CuO_4 square in the T'-phase is considerably expanded and the c axis shrunk, relative to the T- and T*-phase structures, for which $a=3.78$ Å, $c=13.2$ Å, and $a=3.85$ Å, $c=12.5$ Å, respectively.

Electron-superconductivity is not restricted to the Nd-based compounds, but is also evident in the T'-phase of Pr- and Sm-based copper oxide compounds Ce-doped and reduced by the same procedure; again the electron concentration must be increased beyond $q=0.20$, and superconductivity occurs at around 20 K. In Fig. 4 we show measurements of the Meissner effect in three different samples, $\text{Ln}_{1.85}\text{Ce}_{0.15}\text{CuO}_{4-y}$ (with Ln representing Pr, Nd and Sm), carried out using a SQUID magnetometer in an applied field of 10 Oe under field-cooled conditions. A substantial Meissner signal was observed in all of the compounds. In particular, the large signal for $\text{Nd}_{1.85}\text{Ce}_{0.15}\text{CuO}_{3.93}$ —more than 25% of the ideal value—

confirms the bulk nature of superconductivity. The observed T_c (24 K) is comparable with those in the corresponding hole-superconducting single-layer copper oxides: $T_c=28$ K in the T*-phase (with Cu-O pyramids) and $T_c=40$ K in the T-phase (with Cu-O octahedra).

The compound $\text{Pr}_{1.85}\text{Ce}_{0.15}\text{CuO}_{4-y}$ (Fig. 4) is the first example of a Pr-based copper oxide superconductor. Hole-doped Pr-based copper oxides, such as the T*-phase compound $\text{Pr}_{2-x-y}\text{Ce}_x\text{Sr}_y\text{CuO}_{4-y}$ (Y. T. *et al.*, manuscript in preparation) and the 1-2-3 compound $\text{PrBa}_2\text{Cu}_3\text{O}_7$ (ref. 5), show neither metallic nor superconducting behaviour. This has been

attributed to the partial trapping of doped holes by Pr ions, or equivalently to the mixed-valent nature of Pr^{n+} ions ($3 < n < 4$). The appearance of superconductivity in $\text{Pr}_{2-x}\text{Ce}_x\text{CuO}_{4-y}$ further implies that the carriers are electrons in this T'-phase compound, because Pr^{3+} cannot be further reduced.

In conclusion, we have demonstrated for the first time that electron-doping in copper oxide compounds having the T'-phase structure, comprising CuO_2 sheets with no apical oxygen, gives rise to superconductivity. This observation may contain important implications for theories of high- T_c superconductivity.

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Unusually simple crystal structure of an Nd–Ce–Sr–Cu–O superconductor

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Recently, a new copper oxide superconductor with an onset temperature of 28 K was found in the Nd–Ce–Sr–Cu–O system¹. The superconducting phase was identified by X-ray diffraction and electron microscopy², and the metal-atom positions are identical with those in the K_2NiF_4 or Nd_2CuO_4 structure³, except for the order of the cations Nd(Ce) and Sr. Here we report the refinement of the crystal structure using neutron powder diffraction. We find that it comprises alternating slabs of the K_2NiF_4 and Nd_2CuO_4 structures, and that all the copper atoms are crystallographically identical, with oxygen in fivefold (square-pyramidal) coordination. In having only one kind of copper site, this new compound is structurally simpler than the other CuO_2 -bearing superconductors such as $\text{YBa}_2\text{Cu}_3\text{O}_7$, and may therefore be particularly helpful in the effort to understand the origins of high-temperature superconductivity.

A sample with the composition $(\text{Nd}_{0.66}\text{Sr}_{0.205}\text{Ce}_{0.135})_2\text{CuO}_{4-\delta}$ was prepared by mixing Nd_2O_3 , SrCO_3 , CeO_2 and CuO in the appropriate ratio. The mixture was pressed into a pellet, reacted in air at 1,150 °C for 15 h, and then cooled to 900 °C. The reacted mixture was pulverized, pressed into a pellet and reheated under the same conditions. Finally, it was annealed at 550 °C for 12 h under a high pressure of O_2 (80 kg cm^{-2}), which, as has been observed before (ref. 2 and Y. Tokura *et al.*, preprint), increases both the superconducting volume fraction and T_c .

Our sample showed an onset of superconductivity, from magnetization measurements, at 22 K. The Meissner signal reached ~12% of the ideal value ($-1/4\pi$) at 8 K.

Initial structural parameters for use in a Rietveld analysis of the neutron data⁴ were obtained by preliminary refinement of X-ray powder diffraction data. The X-ray data were measured

Table 1 Structure parameters of $(\text{Nd}_{0.66}\text{Sr}_{0.205}\text{Ce}_{0.135})_2\text{CuO}_{4-\delta}$

Atom	Site	x	y	z	g	$10^3\beta_{11}$	$10^3\beta_{22}$	$10^4\beta_{33}$	B ($\text{\AA}^2$)
M	2c	0	$\frac{1}{2}$	0.3895 (3)	1	—	—	—	0.47 (7)
M'	2c	0	$\frac{1}{2}$	0.1038 (3)	1	—	—	—	0.64 (7)
Cu	2c	$\frac{1}{2}$	0	0.2510 (3)	1	7 (1)	7 (1)	10 (2)	0.48
O(1)	4f	0	0	0.2374 (3)	1	11 (2)	17 (2)	24 (3)	1.05
O(2)	2c	$\frac{1}{2}$	0	0.4288 (5)	0.93 (2)	83 (5)	83 (5)	12 (4)	3.55
O(3)	2a	0	0	0	1	6 (2)	6 (2)	24 (4)	0.76

Estimated standard deviations in parentheses refer to the last digit. The temperature factor is defined as $\exp(-h^2\beta_{11} - k^2\beta_{22} - l^2\beta_{33})$. The occupation factors, g, of Nd, Ce and Sr are fixed at 0.49, 0.1 and 0.41 for site M, and at 0.83, 0.17 and 0.0 for site M'. Isotropic thermal parameters, B, were refined for sites M and M'; equivalent isotropic thermal parameters, B_{eq} , are given for sites Cu, O(1), O(2) and O(3).

Table 2 Interatomic distances, r, in $(\text{Nd}_{0.66}\text{Sr}_{0.205}\text{Ce}_{0.135})_2\text{CuO}_{4-\delta}$

Bonds	r ($\text{\AA}$)	N*
M–O(1)	2.706 (2)	4
M–O(2)	2.7707 (6)	4
M–O(2†)	2.268 (4)	1
M'–O(1)	2.550 (2)	4
M'–O(3)	2.3231 (9)	4
Cu–O(1)	1.9355 (2)	4
Cu–O(2)	2.221 (3)	1

* Number of equivalent bonds.

† Coordinate triplet: y, 1–x, 1–z.

at room temperature using a Rigaku diffractometer with rotating-anode source and graphite monochromator. Intensity data were collected with Cu $K\alpha$ radiation at 0.02° intervals. The diffraction pattern indicated that the sample was nearly free from impurities. Rather low R factors were obtained: $R_{wp} = 12.9\%$, $R_p = 9.8\%$, $R_1 = 8.6\%$, $R_F = 5.9\%$ and $R_e = 7.8\%$. The X-ray data were sufficient to determine the arrangement of the metal ions; a structure refinement on neutron powder diffraction data was carried out to obtain reliable structure parameters for oxygen.

The neutron diffraction data were obtained on a high resolution time-of-flight neutron powder diffractometer, HRP, at the KENS pulsed spallation neutron source at the National Laboratory for High Energy Physics. The specimen was contained in a cylindrical vanadium cell. The scattering angle, 2θ , was fixed at 170°, and intensity data were collected at room temperature for 20 h, using a gate width of 4 μs . Intensity data corresponding to interplanar spacings, d, between 0.51 and 3.37 $\text{\AA}$ (7,012 points) were used for Rietveld refinements. Preferred orientation was corrected on the assumption that the cleavage plane is (001), using a gaussian preferred-orientation function.

The observed data are consistent with just one of the three models proposed by Takayama-Muromachi *et al.*². Final structure parameters are listed in Table 1, and selected metal–oxygen distances in Table 2. The refined lattice parameters are $a = 3.8563$ (3) $\text{\AA}$ and $c = 12.4842$ (9) $\text{\AA}$. R factors were $R_{wp} = 5.9\%$, $R_p = 4.5\%$, $R_1 = 3.5\%$, $R_F = 2.7\%$ and $R_e = 4.8\%$. Figure 1 shows the final profile fit and difference patterns. The good agreement between observed and calculated patterns strongly supports the proposed structural model.

Because the occupation factor, g, and thermal parameters for site O(2) are considerably correlated, these parameters cannot be determined very accurately. However, taking $g = 1$ for site O(2) gave larger R factors and a larger thermal parameter ($B_{eq} = 4.26$), suggesting that this site is slightly oxygen-deficient. The oxygen stoichiometry $4 - \delta$, calculated from the measured g, is 3.93. The increase in superconducting volume fraction and in T_c that accompanies oxygen annealing presumably results from an increase in occupation of this site.

The equivalent thermal parameter of site O(2) was fairly large: $B_{eq} = 3.55 \text{\AA}^2$. The anisotropic thermal parameters U_{11} and U_{33}

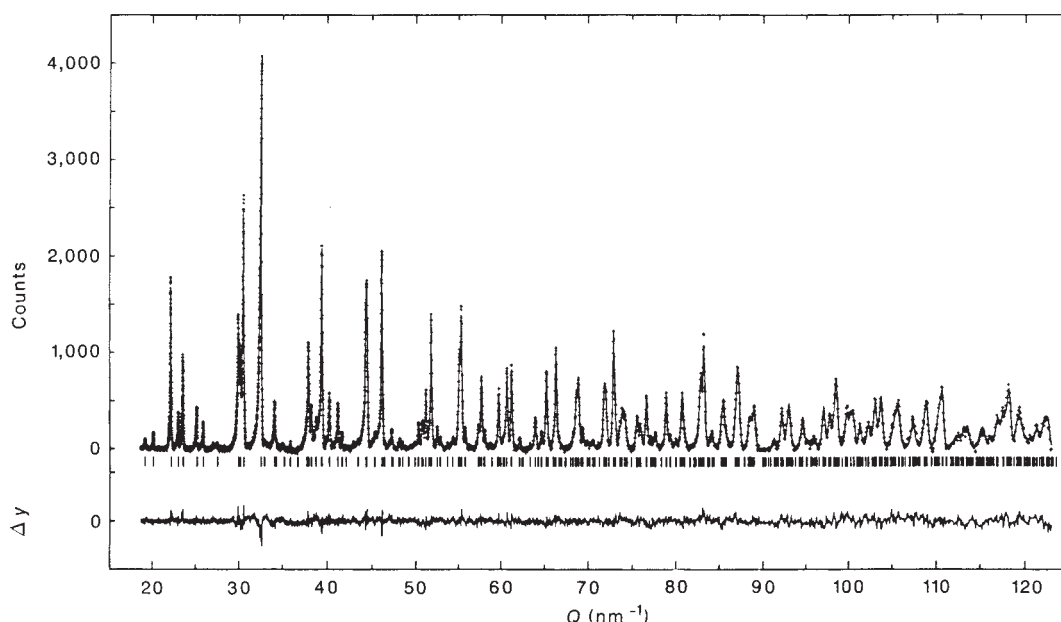


Fig. 1 Rietveld refinement of a time-of-flight neutron diffraction pattern for $(\text{Nd}_{0.66}\text{Sr}_{0.205}\text{Ce}_{0.135})_2\text{CuO}_{4-\delta}$. Scattering vector $Q = 2\pi/d$, where d is the interlayer spacing. The solid lines are calculated intensities, crosses indicate experimental data, and Δy is the difference between the two. The short vertical lines mark the positions of 501 peaks.

are, respectively, $0.063 \text{ \AA}^2$ and $0.0092 \text{ \AA}^2$, indicating that the thermal ellipsoid of O(2) expands perpendicular to the c axis. This marked anisotropy of thermal vibration is understandable in view of the relatively short Cu-O(2) and M-O(2) bonds parallel to the $[001]$ direction. In addition, the large value of U_{11} for this site must be partly due to the random distribution of Nd, Ce and Sr atoms at site M.

The coordination numbers of M, M' and Cu are, respectively, 9, 8 and 5. Sr^{2+} ions, which are larger than Nd^{3+} or Ce^{3+} (or Ce^{4+}) ions, selectively occupy the 9-coordinate site at $(0, \frac{1}{2}, \sim 0.39)$. The average M-O distance ($2.69 \text{ \AA}$) is much larger

than that of M'-O ($2.44 \text{ \AA}$). The CuO_5 pyramid contains four short Cu-O(1) bonds ($1.9355 \text{ \AA}$) and one long Cu-O(2) bond ($2.221 \text{ \AA}$), but the Cu-O(2) bond is shorter than those in other superconductors with such CuO_5 pyramids, such as $\text{YBa}_2\text{Cu}_3\text{O}_7$ and $\text{Tl}_2\text{CaBa}_2\text{Cu}_2\text{O}_8$. The valence of Cu, calculated using the Brown-Altermatt formula⁵, is 2.23. The bases of the pyramids form a two-dimensional CuO_2 plane perpendicular to the $[001]$ direction. The M-O(2) bond parallel to the $[001]$ direction is only $2.268 \text{ \AA}$, which is comparable with the Cu-O(2) distance.

The crystal structure of $(\text{Nd}_{1-x}\text{Sr}_x\text{Ce}_y)_2\text{CuO}_{4-\delta}$ is shown in Fig. 2. The unit cell consists of two parts: the upper part in Fig. 2 corresponds to the K_2NiF_4 structure, in which the M (Nd, Ce, Sr) ion is coordinated to nine oxygen atoms, and the lower part exhibits the Nd_2CuO_4 structure, incorporating M' (Nd, Ce) ions in eightfold coordination. All of the copper atoms are in a single crystallographic site, with square-pyramidal coordination. High- T_c superconductors are classified into two structural groups, those that contain copper in distorted octahedral coordination (for example, $(\text{La}_{1-x}\text{M}_x)_2\text{CuO}_4$ (where M is Ca, Sr or Ba) and $(\text{Bi}, \text{Tl})_2(\text{Sr}, \text{Ba})_2\text{CuO}_6$) and those that contain copper in pyramidal coordination (for example, $\text{LnBa}_2\text{Cu}_3\text{O}_7$ (where Ln is Y or various lanthanide elements), $(\text{Bi}, \text{Tl})_m\text{Ca}_n(\text{Sr}, \text{Ba})_{2-n-m}\text{Cu}_{n+1}\text{O}_{m+2(n+2)}$ and $\text{Pb}_2\text{Sr}_2\text{ACu}_3\text{O}_{8+\delta}$ (where A represents Ln or $\text{Ln} + (\text{Sr} \text{ or } \text{Ca})$)⁶). Clearly, $(\text{Nd}_{1-x}\text{Sr}_x\text{Ce}_y)_2\text{CuO}_{4-\delta}$ has the simplest structure of the 'pyramidal' superconductors.

Here we have assumed that Ce^{3+} ions occupy both the M and M' sites. We are now examining the local and electronic structures of Ce by extended X-ray absorption fine structure (EXAFS) and X-ray photoelectron spectroscopy (XPS), with the aim of determining the distribution of Ce between these two sites. We are also investigating the apparent sensitivity of superconductivity to the Nd/Sr/Ce ratio.

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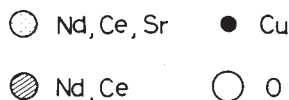
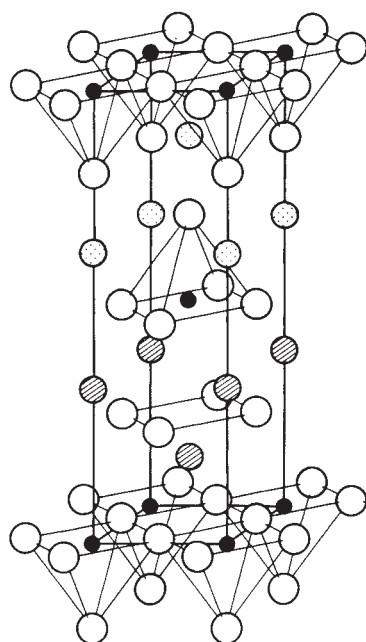


Fig. 2 Crystal structure of $(\text{Nd}_{1-x}\text{Sr}_x\text{Ce}_y)_2\text{CuO}_{4-\delta}$.

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Experimental and theoretical equation of state of cubic boron nitride

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The phase diagram of boron nitride (BN) is similar to that of carbon, incorporating phases at high temperatures and pressures whose structures and physical properties resemble diamond¹⁻⁹. Cubic zinc-blende-structured BN is especially important because it is extremely hard—second only to diamond. Here we report the first measurement of the 300-K equation of state of this material to ultrahigh pressures (115 GPa), and obtain a zero-pressure bulk modulus of 369 ± 14 GPa. A theoretical equation of state derived from first-principles pseudopotential calculations yields a 300-K isotherm that agrees with our experimental results to better than 2.5% in volume and 2.0% in bulk modulus. The high-pressure Hugoniot (shock-wave equation of state) calculated from our equation of state for BN is in good agreement with existing shock-wave data. Our study illustrates the reliability of current experimental techniques (such as the ruby-fluorescence calibration) and theoretical methods (pseudopotentials) for characterizing the behaviour of superhard, incompressible materials under high pressure.

At ambient conditions, BN exists in a hexagonal structure which is analogous to that of graphite. At pressures above 4.5 GPa and temperatures above 1,500 K, BN exhibits two polymorphs, corresponding to the diamond (cubic zinc-blende-structured) and lonsdalite (hexagonal wurtzite-structured) forms of carbon. Both the structure and the physical properties of zinc-blende-structured BN, the thermodynamically stable phase at high pressure, resemble those of diamond. Because of its extreme hardness, its chemical inertness, its high melting temperature ($>3,000$ K) and its high thermal conductivity (~ 200 W m⁻¹ K⁻¹), cubic BN is commercially important¹⁰. But despite these unusual physical properties, there has been no direct experimental measurement of its equation of state. This lack of data is surprising because the value of the bulk modulus, for example, is of considerable importance in understanding the relationship between a crystal's structure, hardness and stability—information that can help in the design of new superhard

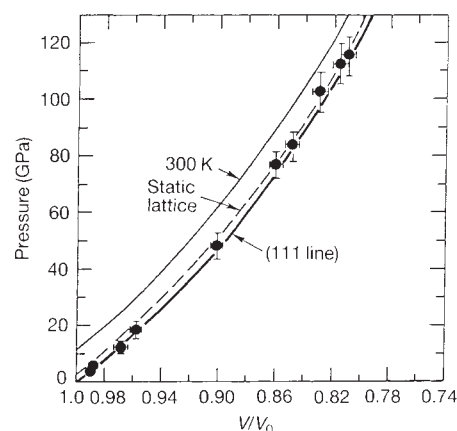


Fig. 1 Experimental pressure-volume data for cubic BN at 300 K (circles with error bars) are compared with theoretical isotherms for the static lattice (dashed curve) and for BN at 300 K (thin solid line). The thick solid line is the Birch-Murnaghan (third-order Eulerian finite strain) isotherm that is obtained from a least-squares fit to our static compression measurements. The reference volume used here is the volume measured at ambient conditions, $V_0 = 7.113$ cm³ mol⁻¹, so the theoretical and experimental results are compared here in terms of absolute volume.

materials with technological potential. In addition, the equation of state offers a powerful way of comparing the reliability and predictive power of current theoretical and experimental techniques for characterizing the physical properties of condensed matter. We have determined the equation of state of cubic BN to pressures in excess of 100 GPa by both experimental and theoretical means.

The sample of cubic BN used in this study was supplied by R. C. DeVries of General Electric Corporation and comprised single crystals 30–50 μ m in size. The lattice parameter, determined by X-ray diffraction at zero pressure, is 3.615 ± 0.002 Å. For the high-pressure experiments, gasketed samples of cubic BN were compressed in a Mao-Bell-type diamond cell between diamonds with 200- μ m culets¹¹. The sample region was typically 100 μ m in diameter and 10 μ m thick at high pressures. A 4:1 mixture of methanol:ethanol was used as a pseudohydrostatic pressure medium, and between two and five small pieces of powdered ruby were included with the BN sample for pressure measurement using the ruby-fluorescence technique^{12,13}. A Mo K α X-ray beam was collimated to a diameter of 80 μ m and directed through the high-pressure samples along the axis of force of the diamond cell. X-ray diffraction patterns were recorded at pressures of between 0 and 115 GPa with typical run times of 100–150 hours.

The experimentally measured lattice parameters and volumes of cubic BN under pressure are listed in Table 1, and the isothermal equation of state is plotted in Fig. 1. To determine the zero-pressure values of the isothermal bulk modulus (K_0) and its pressure derivative (K'_0), we have analysed the data using a Birch-Murnaghan (third-order Eulerian finite-strain) equation of state¹⁴. Despite the large value of K_0 (369 ± 14 GPa), we have determined it and K'_0 with considerable precision because of the high pressures that were reached¹⁵.

The static structural properties of cubic BN were calculated using a first-principles total-energy approach¹⁶ within a localized-orbital formalism¹⁷. The localized basis provides a more convenient representation than a plane-wave basis, which had been used in a previous calculation¹⁸, because the valence states of BN are highly localized by the large depth of the angular-dependent components of the boron and nitrogen pseudopotentials. The formalism that we used is described in detail elsewhere^{16,17}, and as far as possible we chose the same approximations here as were used before¹⁸⁻²². To facilitate the evaluation

Table 1 High-pressure X-ray diffraction data for cubic BN

Run no.*	Pressure (GPa)	Lattice parameter† (Å)	Volume V/V_0
090287a	3.2 (± 0.2)	3.605 (± 0.002)	0.992 (± 0.001)
090987a	6.5 (± 0.4)	3.594 (± 0.003)	0.983 (± 0.002)
091587a	12.5 (± 2.1)	3.574 (± 0.006)	0.967 (± 0.004)
092587a	17.9 (± 3.0)	3.564 (± 0.005)	0.958 (± 0.004)
101187b	48.0 (± 4.2)	3.492 (± 0.006)	0.901 (± 0.005)
102187b	76.5 (± 4.7)	3.438 (± 0.006)	0.860 (± 0.005)
111787b	82.8 (± 5.2)	3.422 (± 0.005)	0.848 (± 0.004)
120887b	101.8 (± 6.8)	3.399 (± 0.006)	0.831 (± 0.005)
123087b	112.0 (± 7.3)	3.378 (± 0.006)	0.816 (± 0.005)
010788b	115.6 (± 7.1)	3.370 (± 0.005)	0.810 (± 0.004)

* a and b designate different samples.

† The lattice parameter is from the (111) diffraction line only. All other diffraction lines of cubic BN have intensities of less than 10% of the intensity of this line and therefore are not strong enough to be observed at high pressure.

of matrix elements, the angular-dependent components up to $l=2$ were expanded in terms of gaussian functions using a Monte Carlo simulated-annealing method²³. Up to 12 gaussians were used in the expansion of the sharp p -potentials, which resulted in fitting errors smaller than 0.01 Rydbergs. The wavefunctions are expanded in a basis of Bloch sums of gaussian orbitals $f(r)$ centred on the atomic sites, which have the form:

$$f(r) = e^{-\alpha r^2} r^l Y_{lm}(\Theta, \Phi)$$

Four and five even-tempered values of α are used for B and N respectively, and values of $l=0$ and 1 are included, so that there are 36 basis functions per molecule. The gaussian decays α for B and N were chosen by fully minimizing the total energy with respect to the smallest and largest α s in the even-tempered series. They are 0.23 and 5.80 for N, and 0.17 and 5.3 for B. Inclusion of states with d -symmetries shifted the energies for the equilibrium volume almost uniformly by ~ 0.1 eV per atom pair. This small change is typical of first-row elements, whose energy separation between the atomic d -states and the Fermi level is very large. The crystal potential is iterated to full self-consistency. To perform the summations in the Brillouin zone, the same ten k -point samplings as used in the earlier calculations were chosen¹⁸.

The results of our calculations on cubic BN are shown in Fig. 2 and summarized in Table 2. The static-lattice energy was obtained at each of 12 volumes between 50 and 90 a.u.³ (1 atomic unit (a.u.) = 0.529 Å). To compare the results with 300-K data, we used the Debye model to derive the zero-point energy and the internal-energy difference between 0 K and 300 K. Our approach in making these vibrational corrections to the lattice energy is purely empirical: we use the experimental values of $\theta_D = 1,800$ K for the Debye temperature and $\gamma_0 = 1.87$ for the Grüneisen parameter^{10,24} referenced to the volume measured at ambient conditions, $V_0 = 79.78$ a.u.³. Also, we assume that γ/V is constant (see, for example, ref. 25). Because of the large θ_D of BN the difference between lattice energies and static-lattice energies is almost entirely due to the zero-point energy, and the effect of compression is to increase the vibrational correction (through the effect on γ) so that the equilibrium volumes at 0 K and 300 K are larger than the volume for the static lattice.

The equilibrium properties derived from the calculations depend in detail on the form of the equation of state that is used to fit the energy-volume points. In Fig. 2 we have assumed that the energy is a third-order polynomial in the Eulerian finite-strain, as we did in our analysis of the experimental measurements¹⁴, because this is identical to fitting pressure-volume data with the Birch-Murnaghan equation of state. Thus, we are treating our theoretical and experimental results identically, iteratively solving to obtain V_0 for the best-fitting equation of energy as a function of strain. We obtain values of 3.625 Å

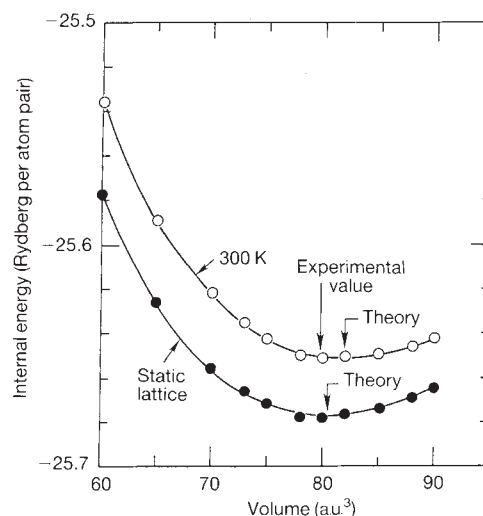


Fig. 2 Theoretical results for the internal energy (Rydberg per molecule) as a function of volume (in atomic units) of cubic BN. The static lattice energies (solid circles) are calculated using the LCAO pseudopotential approach. Corrections for zero-point energy and for the thermal energy between 0 K and 300 K were obtained from the Debye model, and the open circles represent the total energy at 300 K. The two curves show Eulerian finite-strain fits to the static-lattice and 300-K points, with the difference in energy between 0 K and 300 K being within the width of the 300 K curve. Arrows labelled Theory indicate the theoretical equilibrium volumes for the static lattice and at 300 K. These are derived from the fitted curves and are compared with the experimentally determined value for the volume at 300 K.

and 3.648 Å for the lattice parameters of the static and 300-K lattice, respectively, which agree with experiment to better than 1% (Table 2).

Our results compare favourably with the previous calculations using a plane-wave basis¹⁸. If a Murnaghan equation of state is used to fit the calculated points, as had been done in the earlier work, the differences between the equilibrium properties calculated for the static lattice for localized and plane-wave bases are small relative to the uncertainties (Table 2). These differences are comparable to the variation caused by changing the form of the equation of state used. In addition, absolute uncertainties are dependent on the convergence achieved and on the formulation used for the exchange and correlation energy (for example, the Hedin-Lundqvist expression²⁶). Nevertheless, the high level of convergence achieved here is reflected in the cohesive energy (Table 2), which is slightly greater than was

Table 2 Comparison of the properties of cubic BN from experiment and theory

	Lattice parameter (Å)	K_0 (GPa)	K'_0	Cohesive energy E_{co} (eV per atom pair)	
Experiment (300 K)					
Static comp. (0–115 GPa)	3.615 (± 0.002)	369 (± 14)	4.0 (± 0.2)	—	Birch fit ¹⁴
Theory (Static lattice: 0 K)					
LCAO*	3.625 (± 0.001)	368	3.6	—	Birch fit ¹⁴
LCAO*	3.618 (± 0.008)	370 (± 10)	3.1 (± 0.1)	13.5 (± 0.1)†	Murnaghan fit ³⁴
PW‡	3.611	367	3.05	13.3	Murnaghan fit ³⁴
Theory (300 K)					
LCAO*	3.648 (± 0.001)	363	3.5	—	Birch fit ¹⁴

* Linear-combination-of-atomic-orbitals basis: this work.

† The experimental value for the cohesive energy of hexagonal BN is 13.0 eV (ref. 32). The cohesive energy for the zinc-blende phase should differ from this by ~ 0.1 eV (ref. 33). The somewhat larger value obtained theoretically is typical of calculations using the local-density approximation.

‡ Plane-wave basis as in ref. 18.

obtained in the plane-wave calculation. Apparently, using a finite number of plane waves in the latter calculations leads to a reduced estimate of the cohesive energy.

In Table 2 and Fig. 1, we compare the experimental and theoretical equations of state for cubic BN. The theoretical isotherms are obtained by adding the zero-point and thermal pressure derived from the Debye model to the negative volume-derivative of the static-lattice energy curve shown in Fig. 2. It is clear that although the calculated volume is slightly (but systematically) large, by 2.0–2.5% relative to the measured values, the theoretical equation of state is in excellent agreement with our experimental isotherm. In fact, the derived parameters K_0 and K'_0 are identical, given the uncertainties and approximations involved (Table 2). We conclude, therefore, that the high-pressure compression of cubic boron nitride is well characterized by these theoretical and experimental results, confirming the predictions of our earlier study¹⁸.

To check the reliability of our equation of state for cubic boron nitride, we compare our results with independent shock-wave (Hugoniot) measurements^{5,27}. The advantage to the latter is that whereas static experiments depend on the ruby-fluorescence technique for calibrating pressures, the Hugoniot data represent direct measurements of volumes and pressures, free from any pressure calibration. However, the primary difficulties involved in interpreting shock-wave data are: (1) the need to apply thermal corrections and to account for initial sample porosity; (2) the possible influence of strength effects; and (3) possible kinetic hindrances in the case of shock-induced phase transitions.

The existing Hugoniot data for BN are obtained from polycrystalline samples that are initially in the low-pressure (hexagonal) polymorph, with variable porosity ranging between 5 and 10%. We calculate Hugoniot assuming that the hexagonal BN transforms entirely to the cubic phase (that is, that there is no kinetic hindrance) and that strength effects can be neglected. If either assumption is wrong, the actual Hugoniot states would be at pressures (or shock-wave velocities, U_s) greater than our calculated values for a given volume or particle velocity (u_p). The calculations are carried out using the Mie–Grüneisen and Eulerian finite-strain formalisms, with both initial porosity and the energy of transformation being taken into account^{25,28}.

Using the parameters listed under Fig. 3, we predict the high-pressure Hugoniot for BN in terms of U_s as a function of u_p . Although we cannot prove that cubic BN, rather than another phase or a mixture of phases, is formed at high shock pressures, the good agreement between the calculated and observed Hugoniot does support this inference. There is no evidence for strength effects or for partial transformation at $u_p > 2 \text{ km s}^{-1}$, except in the pyrolytic samples of ref. 5. Even the curvature of the U_s – u_p trend is reproduced by our calculations (Fig 3). According to our model this curvature is due mainly to the initial sample porosity, although the experimental curvature may exceed that predicted above $u_p = 4.2 \text{ km s}^{-1}$. We concur with Gust and Young⁵ that the samples may be molten at these high shock pressures. For example, we estimate Hugoniot temperatures of 5,000–5,500 K at 100 GPa for the porous samples shown in Fig. 3; these values of temperature are probably low because they are obtained assuming that the specific heat is equal to the Dulong–Petit value⁵. Hence, shock-induced melting is a plausible explanation for the observed U_s being somewhat lower than our predicted values at Hugoniot pressures above 85 GPa ($u_p > 4.2 \text{ km s}^{-1}$). As a result, we conclude that the shock-wave data are in good agreement with the equation of state that we have derived by static-compression measurements and by linear-combination-of-atomic-orbitals pseudopotential calculations. This provides implicit additional support for the validity of the ruby-fluorescence calibration used in static-compression experiments.

Previous empirical estimates of the bulk modulus of cubic BN range up to $465 \pm 50 \text{ GPa}$. This value, based on the interpola-

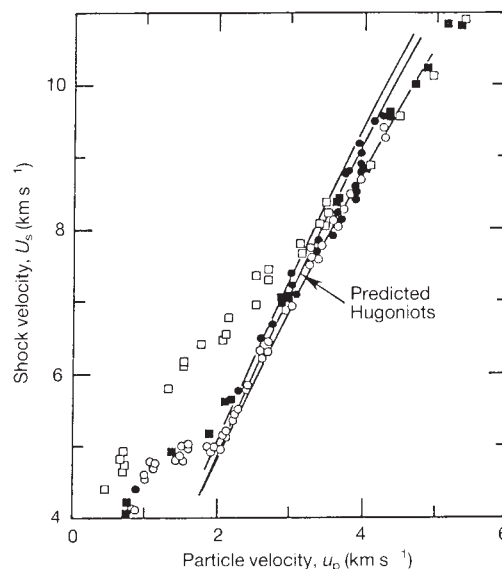


Fig. 3 Comparison of shock-wave data on BN with Hugoniot predicted from the present equation of state for cubic boron nitride. Open and closed squares represent the pyrolytic and hot-pressed samples of ref. 5, whereas the open and closed circles represent samples of ref. 28 with initial densities of 2.08 Mg m^{-3} and 2.11 – 2.15 Mg m^{-3} , respectively. The middle curve corresponds to the Hugoniot derived from the following parameters (notation as in ref. 28): $\rho_{00} = 2.10 (\pm 0.05) \text{ Mg m}^{-3}$ (refs 5, 35), $\rho_{01} = 2.258 (\pm 0.001) \text{ Mg m}^{-3}$ (ref. 35), $\rho_{02} = 3.489 (\pm 0.001) \text{ Mg m}^{-3}$ (ref. 35), $K_{s02} = 371 (\pm 14) \text{ GPa}$, $dK_{s02}/dP = 4.0 (\pm 0.2)$, $\gamma_{02} = 1.87 (\pm 0.322)$ (refs 10, 24), $d \ln \gamma / d \ln V = 1$ and $E_{tr} = 5 (\pm 3) \text{ kJ mol}^{-1}$ (ref. 10). The outer curves reflect the estimated uncertainties in the Hugoniot parameters.

tion of empirical correlations for elastic constants, is clearly too high²⁹. In contrast, a scaling argument in which the bulk modulus for diamond- and zinc-blende-structured compounds is inversely proportional to a power of the bond length leads to an estimate of 367 GPa for cubic BN (ref. 30). This is in close agreement with both our experimental and theoretical values of K_0 , and it gives a prediction of K'_0 that is close to our observed value of 4 (ref. 31). Furthermore, the scaling argument successfully reproduces the bulk moduli of a number of diamond- and zinc-blende-structured solids, including carbon (435 GPa), silicon (98 GPa) and germanium (76 GPa). The success of this scaling is consistent with the idea that shorter bond distances and smaller heteropolarities are associated with larger bulk moduli, and hence larger bond strengths and greater hardness, in tetrahedrally bonded solids.

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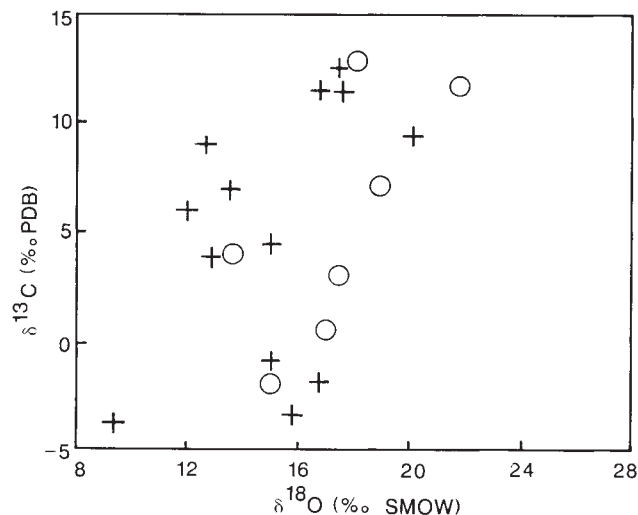


Fig. 1 $\delta^{13}\text{C}$ (relative to PDB) versus $\delta^{18}\text{O}$ (relative to standard mean ocean water (SMOW)) for analysed calcites. Circles represent Loch Maree data, and crosses are Gairloch data.

Evidence from Lewisian limestones for isotopically heavy carbon in two-thousand-million-year-old sea water

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A number of 2,000-million-year-old marbles have extraordinarily heavy carbon isotope signatures ($\delta^{13}\text{C} \approx +12\%$ PDB)^{1,2}, suggesting that there was a worldwide excursion in the carbon isotope composition of sea water at this time. To test this hypothesis we have measured the carbon isotope signatures of 2,000-million-year-old amphibolite facies marbles from the Scottish Lewisian. The occurrence of a major isotope excursion is confirmed by the heavy carbon isotope signatures of these rocks ($\delta^{13}\text{C}$ up to $+13\%$ PDB), which we attribute to an origin contemporaneous with sedimentation. This excursion coincides with a major change in the redox state of the oceans which is likely to have induced considerable changes in the carbon cycle. We suggest that the excursion was due to increased rates of organic carbon deposition resulting from an increase in organic productivity.

Supracrustal rocks from the Loch Maree Group of the Lewisian were deposited after the main 2.7-Gyr granulite facies metamorphic event. These rocks include marbles, graphite schists, iron formations and quartzofeldspathic gneisses which underwent amphibolite facies metamorphism at 1.8 Gyr. The depositional age is unlikely to exceed 2.1 Gyr on the basis of the low initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio at the age of metamorphism³, and of Sm–Nd crustal-residence ages⁴. The marbles include coarsely crystalline and microbrecciated varieties. Both quartz-present assemblages and quartz-absent, dolomite-bearing (such as tremolite–dolomite–calcite⁵) assemblages occur. Carbon and oxygen isotope compositions of calcites extracted from these marbles are shown in Fig. 1 and Table 1. A range of $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values is found which may be attributed to interaction of marbles, initially containing heavy carbon and oxygen, with varying amounts of metamorphic fluid. Many samples containing heavy carbon also preserve oxygen isotope compositions similar to pre-metamorphic values. This is good evidence for a pre-metamorphic origin of the heavy carbon isotope signature, as any process involving a carbonate during metamorphism is likely to decrease its $\delta^{18}\text{O}$ value. Exchange with other silicates,

exchange with an infiltrating fluid and devolatilization reactions are all likely to cause decreases in $\delta^{18}\text{O}$. Hence high- $\delta^{18}\text{O}$ calcites must have been little affected by metamorphic processes and should therefore preserve pre-metamorphic $\delta^{13}\text{C}$ (see, for example, ref. 6). In general, it is hard to conceive of metamorphic processes likely to increase $\delta^{13}\text{C}$ of a marble by more than a few parts per thousand. Metamorphism normally results in decreases in calcite $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ (ref. 6). We conclude that marbles from the Lewisian had $\delta^{13}\text{C}$ values of $\sim 12\%$ over a large area and that this signature is attributable to some synsedimentary process.

During the Phanerozoic, carbon isotope variations in marine limestones did not generally deviate by more than about 4‰ from the present $\delta^{13}\text{C}$ value of 0‰ (ref. 7). There is evidence, however, that during certain periods within the Precambrian many limestones had $\delta^{13}\text{C}$ values in excess of $+5\%$ (refs 8–12). A summary of carbon isotope variations in Precambrian marine limestones is shown in Fig. 2. Further data, not plotted in Fig. 2, exist for the period 1.0–0.5 Gyr which supports the occurrence of a series of positive excursions in seawater $\delta^{13}\text{C}$ at this time¹². Of particular note are the extraordinarily ^{13}C -enriched marbles from the Lomagundi Province of Zimbabwe. Here dolomitic marbles associated with graphite-rich metasediments have isotope compositions of up to $\delta^{13}\text{C} = +12\%$. These marbles were deposited between 1.95 and 2.65 Gyr ago¹. Recently, ^{13}C -enriched marbles with $\delta^{13}\text{C}$ of up to $+12\%$ have been found in Lofoten–Vesterålen, Norway. These were deposited between 1.8 and 2.1 Gyr ago in association with graphite schists and iron formations¹³. The marbles have experienced amphibolite facies metamorphism, but the heavy carbon isotope signatures have been attributed to pre-metamorphic processes on the basis of arguments identical to those above (ref. 2 and A.J.B. and A.E.F., manuscript in preparation). Heavy carbon isotope signatures have also been recorded in 2.0-Gyr-old marbles from Finland (ref. 8 and J. Karhu, personal communication) and in marbles of similar age from Soviet Karelia¹⁴. Interpretation is hampered by the poor resolution of the geochronological data (see Fig. 2), but it appears that ^{13}C -enriched limestones were particularly common about 2.0 Gyr ago. It is not clear whether this represents a single positive excursion in $\delta^{13}\text{C}$ or a series of excursions analogous to those described for the period 1.0–0.5 Gyr (ref. 12). The latter excursions are associated with changes in the $\delta^{34}\text{S}$ of marine sulphate. This may be explained in terms of maintenance of the redox cycle of the hydrosphere by the carbon and sulphur cycles¹⁵. A sulphur isotope excursion has not been

observed for the 2.0-Gyr $\delta^{13}\text{C}$ anomaly^{11,16}. At this time, however, the oxygen cycle may have been controlled by coupling between the carbon and iron cycles rather than between the carbon and sulphur cycles¹⁵. Secular variations in the carbon isotope ratios of marine carbonates between 1.0 and 2.0 Gyr are as yet poorly constrained; this may be a period during which sea water had 'normal' carbon isotope signatures close to 0‰.

The 2.0-Gyr excursion could be attributed to a number of processes. It could represent a worldwide excursion in seawater carbon isotope composition, excursions in sea water restricted to localized basins or the operation of some process restricted to particular intervals within the geological record. Today, ^{13}C -enriched carbonates may be deposited from CO_2 derived from zones of anaerobic diagenesis¹⁷ or from hot spring waters¹⁸. The former process is unlikely to explain the isotope systematics of carbonate-rich sediments and the latter is unlikely to explain the deposition of carbonate over large areas, restricted to particular intervals of geological time. Moderately ^{13}C -enriched carbonates are found in some evaporitic environments¹⁹. Evaporites are not, however, restricted to particular intervals within the Precambrian²⁰. Sedimentary sulphates do occur as accessory minerals in some Lewisian and Lomagundi sediments, but extensive evaporite deposits are absent.

The carbon isotope compositions of marine limestones are generally controlled by the proportion of seawater carbon that passes into limestones and the proportion that passes into organic carbon (see, for example, refs 12, 21). Increases in the amount of organic carbon deposited can lead to positive $\delta^{13}\text{C}$ excursions, as during Phanerozoic anoxic events (ref. 22, for example). High- $\delta^{13}\text{C}$ limestones from Lomagundi, the Lewisian and Lofoten-Vesterålen are associated with organic-carbon-rich metasediments. We suggest therefore that the positive anomaly is associated with an increase in organic-carbon deposition in sediments either on a worldwide basis or within many marginal basins. Return to the deposition of carbonates with more normal isotope compositions could have been achieved by the operation of a number of buffering processes, perhaps including an increase in the rate of organic-carbon recycling within the oceans.

It appears that high- $\delta^{13}\text{C}$ carbonates deposited ~2 Gyr ago were deposited approximately synchronously with the transition from a reducing to an oxidizing hydrosphere²⁰. The end of

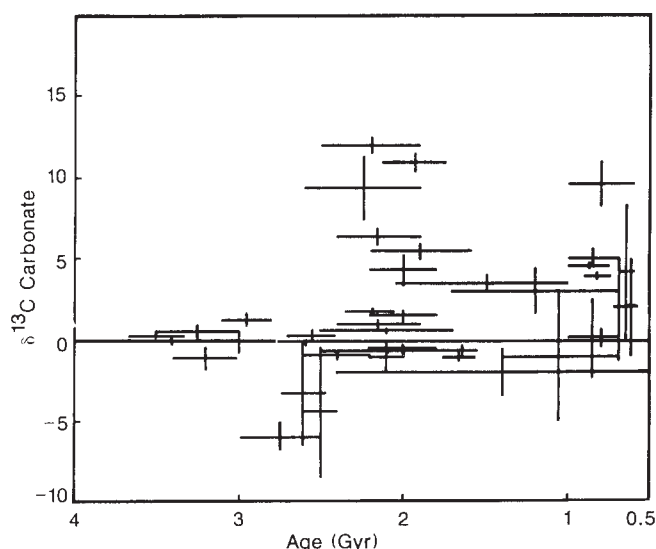


Fig. 2 $\delta^{13}\text{C}$ of Precambrian sedimentary carbonates plotted against age of deposition. Data from refs 1, 2, 8, 9, 10, 11 and this study.

uraninite-conglomerate deposition and changes in the oxide minerals found in fluvial deposits and palaeosols suggest that a large increase in quantities of atmospheric oxygen occurred between 2.5 and 2.0 Gyr ago²⁰. The maximum rate of deposition of iron formations occurred in this period, but declined sharply thereafter, reflecting the transition to a more oxidizing environment²⁰. It has been suggested that similar increases in atmospheric oxygen accompanied the deposition of ^{13}C -enriched limestones between 1.0 and 0.5 Gyr (ref. 12). Increases in atmospheric and oceanic oxygen at about 2.0 Gyr are likely to have been accompanied by major changes in the carbon cycle. We suggest that increased oxygen contents of the oceans were produced and were accompanied by an increase in organic productivity. The increased oxygen content of the atmosphere may have been the direct result of an increase in the population of photosynthesizing bacteria²³. Increasing oceanic oxygen contents may also have enabled the further exploitation of certain oceanic environments and enlargement of the biomass. This increased organic productivity, in the absence of compensating increases in the rate of organic-carbon recycling, would have increased rate of organic-carbon deposition and resulted in the deposition of high- $\delta^{13}\text{C}$ limestones.

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Table 1 Calcite isotope data for Loch Maree Group supracrustals

Specimen number	National grid reference	$\delta^{13}\text{C}$ (% PDB)	$\delta^{18}\text{O}$ (% SMOW)
Loch Maree area			
S72588	NH012656	4.0	13.6
S72591	NH996671	-1.4	14.8
S72600	NG896768	0.5	16.9
S72601	NG903768	12.9	18.0
S72604	NG944760	11.7	22.0
S72605	NG944760	7.2	18.9
S72606	NG944760	3.1	17.5
Gairloch area			
S72609	NG866731	-3.9	9.4
S72613	NG856722	5.8	11.8
S72614	NG856722	3.9	12.8
S72615	NG818720	12.0	17.5
S72616	NG818720	12.1	17.7
S72617	NG838700	8.8	12.8
S72618	NG838700	12.8	17.5
S72620	NG836702	4.7	14.8
S72621	NG828712	7.2	13.6
S72622	NG811725	9.4	21.0
S72627	NG847716	-3.3	15.7
S72632	NG837730	-1.6	16.9

S numbers are British Geological Specimen numbers.

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Relationships between pre-rift structure and rift architecture in Lakes Tanganyika and Malawi, East Africa

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Continental rift systems are rips in plates caused by focusing of extensional stresses along some zone. In the same way that tensile cracks in the side of a brick building generally follow the mortar between bricks, rifts initially follow the weakest pathways in the pre-rift materials. There has even been a suggestion that the occurrence of rifts is controlled by pre-rift structure¹⁻³, although many workers do not subscribe to such direct causality. The relationships between pre-rift structure and rifting tend to become progressively more obscure as the scale of examination decreases, mainly because unravelling these relationships requires more precise information about the architecture of rifts, the nature of the pre-rift fabric and about the principal axes of stress than is generally available. Recent work by Project PROBE on the Tanganyika⁴⁻¹⁵ and Malawi Rift Zones^{12,16-22} permits crucial aspects of these inter-relationships to be examined in a more rigorous fashion than was previously possible. In this region, the rift system follows Proterozoic mobile belts and bifurcates around the Tanganyika craton, which apparently has acted as a resistant core (Fig. 1). Here we show that the fundamental architecture of the Tanganyika and Malawi Rift Zones (that is, the positioning of half-grabens, the way they link together, and the types and trends of linking structures) is strongly influenced by the nature of the pre-rift fabric and the orientation of the stress field.

Fault-pattern maps of the Lakes Tanganyika and Malawi Rift Zones are shown in Fig. 2, and generalized structure maps in Fig. 3. Both rift zones are composed of basins that are asymmetric in cross-section. The deep sides of the basins are bounded by border-fault systems (BFSs), along which subsidence tends to be cumulatively greater than elsewhere in the basin. In plan view the BFSs are mapped as slightly to markedly arcuate. Subsidence varies along BFSs, creating an asymmetry in the strike direction that complements the dip asymmetry. These asymmetric basins, or 'half-grabens'^{8,11,23}, link together in various ways.

The general theme of linking is that adjacent half-grabens are offset from each other in the strike dimension and face in opposite directions, often resulting in a roughly sinusoidal alternation of dip polarities along strike⁴⁻⁸. The best examples of this occur between the Livingstone-Usisya and Usisya-Metangula half-grabens of Malawi. The BFSs of the Usisya and Metangula half-grabens are only slightly offset from each other and the BFSs of the Livingstone and Usisya units virtually merge end-to-end (Fig. 3). One variation on this general theme is for half-grabens to face each other imperfectly, like a pair of vertically skewed parentheses (this is exemplified by the North and East Kigoma half-grabens of Tanganyika, Usisya and the

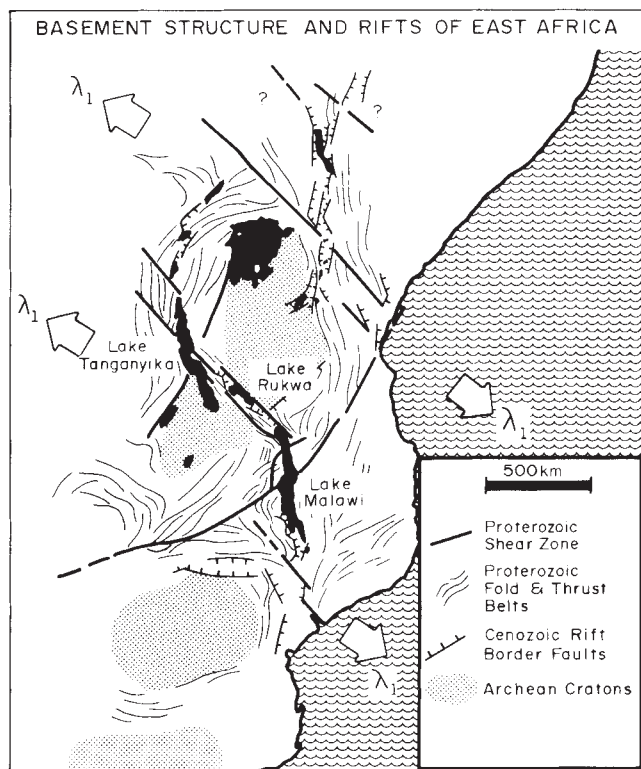


Fig. 1 Tectonic framework of East Africa.

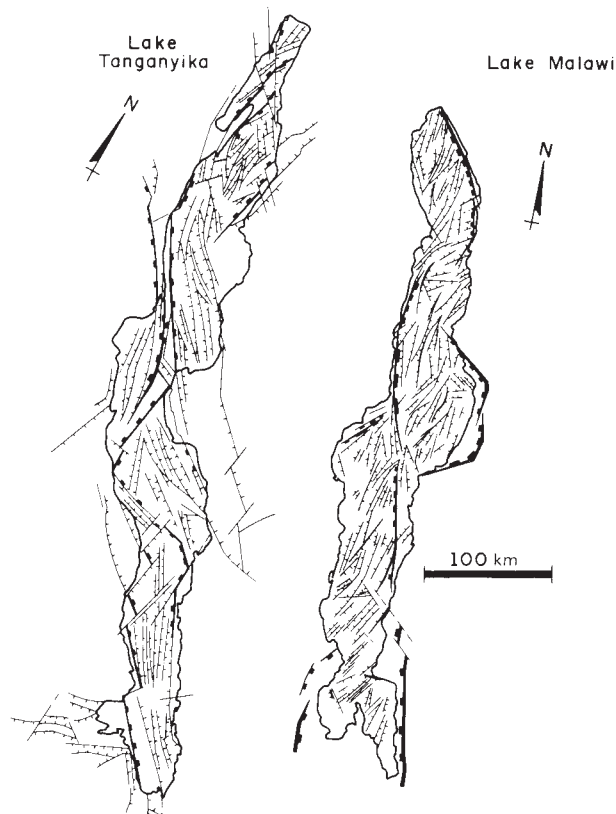
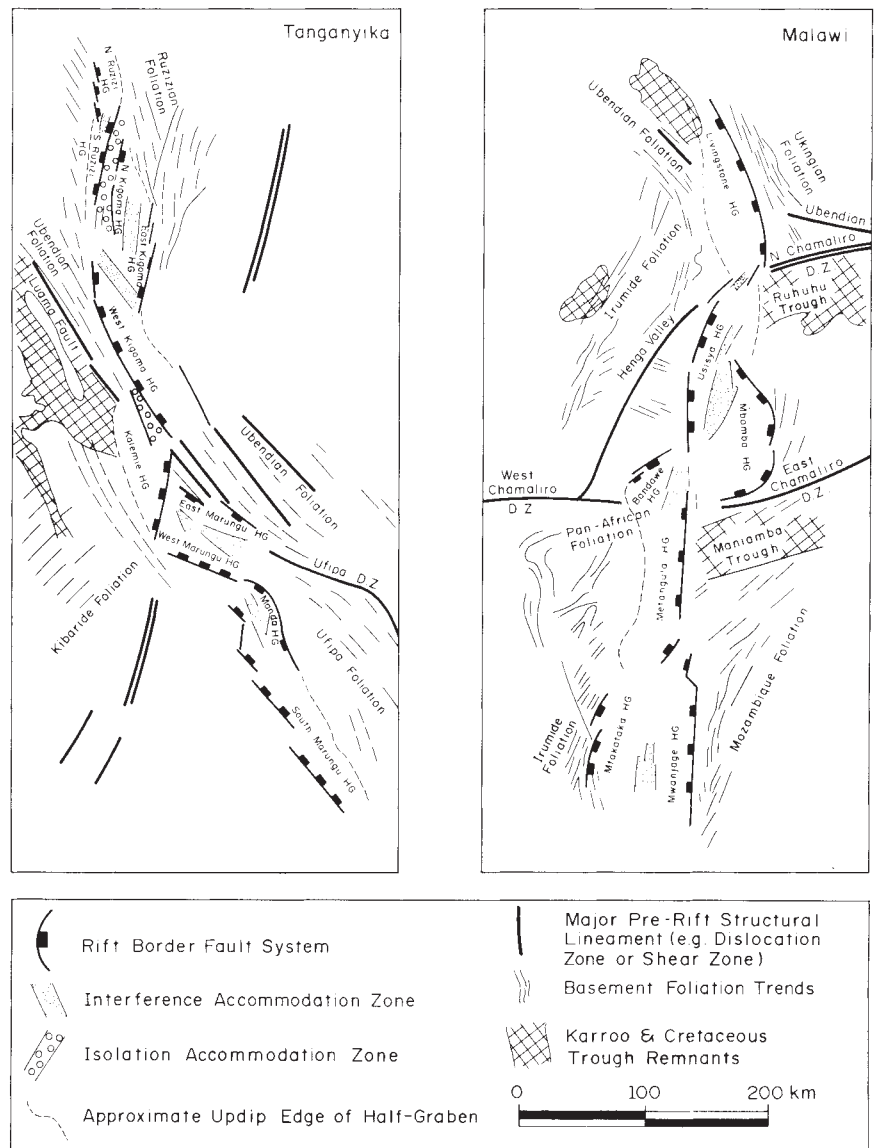


Fig. 2 Detailed fault-pattern maps of the Tanganyika and Malawi Rift Zones, derived from analyses of multifold seismic data. Faults shown are those which offset acoustic basement. Heavy hachured faults refer to master border-fault complexes.

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Fig. 3 Generalized structure maps of the Tanganyika and Malawi Rift Zones superimposed on pre-rift structure. The Tanganyika fabric is that described by McConnell and shown in the UNESCO maps of Africa^{1-3,41,42}, supplemented with more recent mapping and satellite image analyses¹². The Malawi fabric is derived from Bloomfield⁴³ and an analysis by Versfelt¹².



Mbamba half-graben of Malawi). The resulting linking structure is called an interference accommodation zone (INAZ)¹¹. Tanganyika has about five such zones, and Malawi has four (Fig. 3). INAZs are usually basement highs along which deformation is concentrated. The exact origin of INAZs is uncertain but their structural relief and tendency to act as strain guides suggest that they express a space limitation, or competition for space, associated with the facing geometries^{8,11}. Another linking arrangement is for half-grabens to face in opposite directions, like a pair of reversed parentheses, with a backbone of relatively unsubsidized terrain separating the half-grabens⁴⁻⁸. This linking structure is termed an isolation accommodation zone (ISAZ)¹¹. Two ISAZs are mapped in Tanganyika, but there are none in Malawi.

The pre-rift structure of East Africa can be divided into the following categories: cratons and mobile belts, earlier rift troughs, transcontinental dislocation zones and basement foliations¹². Cratons are thick, stable, Archaean nuclei composed of greenstone belts sandwiched between ensialic plutons. Mobile belts are gently arcuate, highly folded Proterozoic rocks that wrap around cratons²⁴. Earlier rift troughs refer to remnants of Permo-Triassic- to Cretaceous-age rift basins. The Ruhuhu and Maniamba Karroo troughs in Malawi trend nearly perpendicular to the lake axis, whereas the Cretaceous trough at the north end of the lake runs essentially parallel to the lake. In

Tanganyika, a Permo-Triassic basin trends sub-parallel to the southern half of the lake and intersects the lake near the central dog-leg (Fig. 3). Transcontinental dislocation zones (TDZs) refer to major sutures in the pre-rift crust that have been activated during various African orogenies and rifting episodes. They generally lie within mobile belts. The most important TDZs in Malawi are the West, East and North Chamaliro zones and the Henga Valley zone, all of which constitute local segments of the Schliesen-Mwimbeshi-Chamaliro TDZ (Figs 1 and 3)^{25,26}. This TDZ may have represented a dextral shear zone during the Pan-African orogeny²⁵, and a sinistral strike-slip fault zone during Karroo times²⁶. The most important TDZ in Tanganyika is the northwest-trending Rukwa dislocation zone (Fig. 1), which connects with the Ufipa zone and Luama Fault (Fig. 3). This zone may have originated during the Ubendian orogeny as a sinistral shear zone^{1,27} and have been reactivated during Permo-Triassic rifting²⁷⁻²⁹. Basement foliation refers to the overall fabric of the pre-rift rocks and includes local faults and fractures, axial planar fabrics, trends of igneous bodies, contrasts in metamorphic grades, schistosity and compositional layer boundaries. Generally, this foliation is the local fabric of the mobile belts in which Tanganyika and Malawi are situated.

Estimates of the regional direction of extension in the Tanganyika-Rukwa-Malawi system range from northwest-southeast through southwest-northeast, but the evidence for

northwest-southeast opening is overwhelming. This extension direction is supported by numerous earthquake studies (refs 30-35 and R. M. Hamilton, unpublished report) and mapping and satellite image analyses (J. Chorowicz, preprint)^{12,26-29,38-43}. Such an opening direction is essentially parallel to the Rukwa, Aswa and Zambesi lineaments, all of which presumably would serve as transform faults^{11,27-29} with continued northwest-southeast opening.

The inter-relationships between rift architecture and pre-rift structure in Tanganyika and Malawi are shown in Fig. 3. The key relationships are summarized in Fig. 4. The most significant relationship is that alternations in half-graben polarity tend to occur where the transcontinental dislocation zones intersect the rift zones. In Malawi the switch in dip asymmetry between the Livingstone and Usisya half-grabens occurs where the Henga Valley-North Chamaliro TDZs cross the rift zone. The polarity switch between the Usisya and Metangula half-grabens occurs where the West Chamaliro-East Chamaliro zone crosses the rift. In Tanganyika the polarity reversal between the West Kigoma and Kalemie half-grabens occurs where the Rukwa-Ufipa-Luama TDZ intersects the rift^{9,10,15}. All three of these intersections and polarity changes are associated with major kinks in the rift zones.

Not all alternations in half-graben polarity are associated with known TDZs, although all known TDZs are associated with alternations. For example, the reversal between the North and South Ruzizi half-grabens in northern Tanganyika (Fig. 3) does not correlate with any known pre-rift dislocation zone. Whether TDZs cause adjacent half-grabens to alternate polarities or whether they simply offer a convenient place for such switch-overs to occur is not certain. We favour the mechanical convenience hypothesis, but readily acknowledge that TDZs could fundamentally alter rift dynamics under certain conditions.

The orientation of a TDZ relative to the rift axis may be related to the type of linking structure observed. The Henga Valley-North Chamaliro dislocation zone in northern Malawi is oriented nearly perpendicular to the inferred extension direction, whereas the Rukwa-Ufipa-Luama TDZ in Tanganyika is essentially parallel. In the latter case, the border fault systems of the adjacent half-grabens splay off the TDZ^{9,10,15} and become more orthogonal to the direction of extension with increasing distance from the TDZ (Fig. 4). In the northern Malawi example, the BFSs also peel off the TDZ, but become more parallel to the extension direction with increasing distance from the TDZ. In the central Malawi case, the Usisya and Metangula BFSs essentially die out as they approach the TDZ at right angles. Here, the extension direction is oblique to both the BFS and the TDZ. These differences may explain the different linking structures observed between the Tanganyika case (ISAZ), the northern Malawi example (very small, narrow INAZ) and the central Malawi situation (broad, diffuse INAZ). Admittedly, the morphology of the latter may also be partly due to the facing geometry of the Bandawe-Mbamba half-graben.

The role of earlier episodes of rifting on the development of the Tanganyika and Malawi Rifts is difficult to isolate from that of the TDZs, because there is a close spatial association between these features. Evaluation of existing data suggests, however, that the rift architectures shown in Fig. 3 would not be significantly different if the earlier rift troughs were not present.

As stated earlier, both the Tanganyika and Malawi Rift Zones generally follow the basic 'grain' of the mobile belts. More surprising is the close association of INAZs with pre-rift fabric (Figs 2 and 3). This relationship is not sensitive to INAZ fault orientations relative to extension direction. Apparently, the fabric of the Proterozoic mobile belts exerts a very strong influence on the orientation of the structures that define INAZs. Careful inspection of Fig. 3 shows that INAZs occur commonly at the intersections between pre-rift fabrics of different trends. The INAZs that divide the North, East and West Kigoma half-grabens of Tanganyika are an example. In effect, INAZs

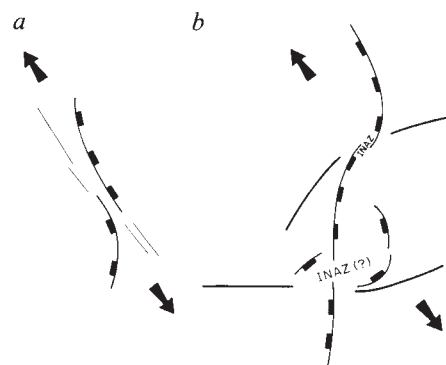


Fig. 4 Selected linking architectures in the Tanganyika (a) and Malawi (b) Rift Zones superimposed on pre-rift structure. Arrows denote inferred direction of extension for the East African Rift. a, Kavala Island model of ISAZ-linked half-graben alternation; the pre-rift shear zones and/or fabric are oriented sub-parallel to the primary extension direction. b, Malawi model of INAZ-linked half-graben alternation; the pre-rift shear zones and/or fabric are oriented sub-perpendicular to the primary extension direction.

divide different basement lithologies and follow the orientation of one of them. This may offer a clue to the origin of INAZs and perhaps also to the factors determining the geometries of facing half-graben.

There also seems to be a general parallelism between the trends of border-fault systems, internal faults and the pre-rift grain, with a few exceptions. One exception occurs where BFSs splay off TDZs and cross pre-rift fabric, as is the case for the West Kigoma and Kalemie BFSs. Another exception is the north-south linear portion of the Usisya-Metangula BFS pair, which seems to cut across the earlier grain. A third exception occurs in the Livingstone half-graben of northern Malawi, where the infrastructure tends northeast-southwest, at about 60° to the trend of the BFS and to the Ubendian and Ukingian foliations (Figs 2 and 3). In spite of the broad correlation between the trends of BFSs and pre-rift fabric, BFSs do not seem to follow major lithological contrasts for appreciable distances¹⁸. This role seems to be reserved for INAZs.

The architectural concepts described for the Tanganyika and Malawi Rift Zones seem to have wide applicability^{4,11}, suggesting that the relationships described herein probably apply elsewhere. We speculate that the polarity reversals that occur in such rifted environments as the Gulf of Suez, Rio Grande Rift and Mid-Continent Rift of North America⁴ are related to the positioning of transcontinental dislocation zones; we hope that this hypothesis will be tested in the future.

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Facultative viviparity in a thrips

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The absence of species that show facultative variation in reproductive mode within populations^{1–4} has thus far severely hindered study of the selective pressures associated with oviparity and viviparity. Here I report that females of the mycophagous thrips *Elaphrothrips tuberculatus* (Insecta: Thysanoptera) are facultatively viviparous and produce male offspring by viviparity and female offspring by oviparity. Individual females can switch between the two reproductive modes. Females produce fewer offspring when viviparous than when oviparous, but the survivorship of viviparous offspring is sufficiently high that the average number of pupal offspring produced by females in each mode is similar. Facultatively viviparous thrips provide the first experimental systems for analysing the behavioural, ecological and physiological causes of variation in reproductive mode.

Elaphrothrips tuberculatus is a winged thrips about 5 mm long that lives on clusters of hanging dead oak leaves and feeds on fungal spores^{5,6}. In southern Michigan, it overwinters as adults of both sexes in leaf litter, emerges in mid-April to mid-May, and undergoes two generations per year. Observations of breeding adults show that some females deposit eggs one at a time, over a 1–3-week period, onto an egg mass which they defend against potential egg predators⁷. By contrast, other females do not lay eggs, although they are gravid during any given breeding period. Dissection of gravid females demonstrates that oviparous females contain four ovarioles with each ovariole comprising enlarging, undeveloped oocytes arranged linearly. Viviparous females contain two clusters of embryos in increasingly advanced stages of development distally, such that fully gravid females contain one or several fully developed first-instar larvae. Two lines of evidence indicate that this variation among breeding females represents a reproductive dimorphism rather

than stages of egg retention: first, the ovaries of more than 738 dissected females were of one or the other type, and second, gravid females isolated in Petri dishes deposit either a mass of undeveloped eggs that they guard ($N = 20$) or several first-instar larvae (with no trace of chorion visible) ($N = 53$). Larvae or eggs are often deposited within 24 h of isolation.

If reproductive mode is under facultative female control, then individual females may be capable of switching between oviparity and viviparity. *E. tuberculatus* females normally reproduce once during the one-month spring generation, but the summer generation lasts for about two months, which allows sufficient time for multiple bouts of reproduction⁶. Allowing females to breed individually in summer on dead oak leaves in nylon bags so as to record their reproductive mode (by noting the presence of egg masses and the presence and sex of offspring^{6,8}) showed that about one-third of females switched reproductive mode in 1986 (21 both modes, 15 viviparous, 20 oviparous) and 1987 (20 both modes, 19 viviparous, 12 oviparous). *E. tuberculatus* is apparently the only animal species known in which individual females are capable of switching between oviparity and viviparity.

Because reproductive mode is under facultative female control in this species, variation in mode may be associated with variation in measurable aspects of females and their environments. The body sizes (lengths of the fore-femora) of viviparous and oviparous females did not differ during the spring generation, but during the summer generation viviparous females were substantially larger than oviparous females⁹. To determine whether or not aspects of seasonality affect female reproductive mode, I allowed individual females from both the spring and summer generation to breed on both spring- and summer-generation dead oak leaves (summer leaves have died within the past few months, whereas spring leaves died in the preceding year). The only treatment in which females were viviparous (5 of 14) was summer females on summer leaves; no females were viviparous in the spring females/spring leaves treatment (0 of 15), the spring females/summer leaves treatment (0 of 13), or the summer females/spring leaves treatment (0 of 14) ($G = 15.4$, $P < 0.005$). These data suggest that a proximate cause of viviparous reproduction involves interaction between a female's generation and the generation of the leaves that she inhabits.

All thrips are believed to have a haplodiploid genetic system, such that haploid males develop parthenogenetically, and diploid females develop from fertilized eggs^{10,11}. Sexing of first-instar larvae^{6,8} showed that, in *E. tuberculatus*, all of 1,051 newly hatched larvae from eggs laid by 42 females were female, and all of 202 larvae produced by 32 viviparous females were male. These data indicate that mode of reproduction is invariably associated with offspring sex. But sperm were seen clearly in the spermathecae of 134 (87%) of 154 dissected viviparous females.

Because females produce single-sex broods, individual breeding females can be considered as units of investment in one sex or the other⁶. Thus, if females are equal in resources available for breeding, then frequency-dependent selection on sex allocation¹² should favour a population-wide proportion viviparity (that is, a sex-allocation ratio) of 50%. In *E. tuberculatus*, deviations from 50% viviparity (Table 1) may be caused by a sex difference in the degree of competition among siblings between spring and summer⁶ and partial bivoltinism with variation between generations in the reproductive value of the two sexes¹³. Moreover, if the large size of viviparous females relative to oviparous females in summer represents a difference in resources available for investment, then the summer sex-allocation ratio is more male biased than it would appear to be from the proportion of females in each reproductive mode.

The association between offspring sex and reproductive mode in *E. tuberculatus* allows testing of a central hypothesis concerning the evolution of viviparity: that viviparous females should produce fewer offspring than oviparous females, but that these

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Table 1 Fecundity of oviparous and viviparous females and the relative survivorship of their offspring

Season	Pupal sex ratio	Mode	Percentage females in mode	Average fecundity	Relative survivorship
Spring	0.19	Viviparity	0.25	21.5	1.4
		Oviparity	0.75	43.7	1.0
Summer	0.58	Viviparity	0.55	13.5	2.1
		Oviparity	0.45	26.1	1.0

Within each sex, the number of pupae present at the end of a generation should be equal to the number of females in the appropriate reproductive mode during that breeding period, multiplied by their average fecundity, multiplied by the survivorship of their offspring from egg laying or larviposition to the pupal stage. Pupal sex ratio was calculated by collecting pupae during the spring and summer generations, allowing them to become adult, and sexing the adults (see refs 6, 9). The spring pupal sex ratio is the average for 1984 (0.19 male, $N = 9,709$), 1985 (0.16 male, $N = 6,617$), and 1986 (0.21 male, $N = 3,158$), and the summer pupal sex ratio is the average for 1985 (0.69 male, $N = 483$) and 1986 (0.46 male, $N = 1,399$) (ref. 6). The percentage of females in each mode was determined by dissecting females and inspecting their ovaries (see refs 6, 9); the percentage for spring is the average for 1986 (0.26 viviparous, $N = 449$) and 1987 (0.24 viviparous, $N = 902$), and the percentage for summer is data from 1986 ($N = 645$). Oviparous female fecundity was measured in spring by counting the number of eggs in complete clutches and taking the average for 1985 (39.5 ± 8.7 , $N = 127$), 1986 (46.6 ± 9.4 , $N = 121$), and 1987 (44.9 ± 9.9 , $N = 145$) (refs 6, 7). In summer, oviparous female fecundity was measured by counting the number of eggs in completed and abandoned egg masses (26.1 ± 11.4 , $N = 222$, data from 1986) (refs 6, 7). In spring, viviparous female fecundity was estimated by counting the number of first-instar larvae produced by individual viviparous females on 2–3 dead oak leaves in the field, dissecting the females near the end of the breeding period, counting the number of embryos in their abdomens, and adding these two numbers; these data (21.5 ± 5.4 offspring, $N = 20$ females) were taken in 1987 (ref. 6). In summer, viviparous female fecundity was measured by putting females individually on 2–3 dead oak leaves in nylon bags suspended in the field early in summer, allowing them to breed for about 2 months, and either: (1) counting the number of adult males that were produced by the females that were viviparous (15.0 ± 6.7 , $N = 15$, data from 1986), or (2) removing and counting the larvae produced every two weeks for eight weeks (12.2 ± 6.9 , $N = 18$, data from 1987) (ref. 6). The value used is the average for 1986 and 1987 (13.5 ± 6.8 , $N = 33$). The relative survivorship, from the egg stage to the pupal stage, of offspring produced by oviparity is set arbitrarily to 1.0, and the relative survivorship of offspring produced by viviparity is obtained by multiplying through.

offspring should have higher survivorship¹⁴. In both generations, the average fecundity of viviparous females was about half that of oviparous females, but the survivorship of the offspring of viviparous females to the pupal stage was 1.4 times higher than that of the offspring of oviparous females in spring and 2.1 times higher in summer (Table 1). The contribution of offspring sex, rather than mode of development, to differences in survivorship is unknown. This hypothesis could be tested more directly using a thrips species, such as *Sporothrips amplius*, in which one sex (males) is produced by both oviparity and viviparity (unpublished data).

In vertebrates, correlates of viviparity include the presence of maternal care in related oviparous species^{3,4} (which is considered as an alternative adaptation to harsh conditions for eggs¹⁵) and the presence of physical defences such as venom or predatory habits (which are presumed to reduce mortality in females encumbered by embryos^{4,16}). In *E. tuberculatus*, maternal care is clearly an alternative to viviparity, and females use a potent defensive secretion, juglone, to repel predators such as salticid spiders (Blum *et al.*, unpublished data). Thrips apparently show a complete range of variation in reproductive mode, including fully oviparous, egg-retaining, facultatively viviparous, and fully viviparous species^{17–19}. Comparative analysis of the correlates of reproductive mode among thrips, coupled with experimental analysis of facultatively viviparous species, should elucidate the selective pressures associated with the two modes of reproduction. Moreover, the presence of facultative viviparity in this taxon suggests that viviparity sometimes originates as a facultative alternative in an otherwise oviparous population, rather than by population-wide directional selection for egg retention.

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A mutation that changes cell movement and cell fate in the zebrafish embryo

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The study of developmental patterning has been facilitated by the availability of mutations that produce changes in cell fate, in animals such as *Caenorhabditis elegans*^{1,2} and *Drosophila melanogaster*^{3,4}. We now describe a zygotic lethal mutation in the zebrafish, *Brachydanio rerio*, that also changes how particular embryonic cells develop. Severe pattern deficiencies are observed that are restricted to a single body region, the trunk. The mutation may directly affect mesoderm, as somites do not form in the trunk. Head and tail structures, including tail somites, are relatively undisturbed. The earliest detected expression of the mutation is during gastrulation, when movements of mesodermal cells occur incorrectly. We injected prospective trunk mesodermal cells with lineage tracer dye and observed that in mutants these cells may enter a new body region, the tail, and there may express a new fate appropriate for the changed position.

The mutation, *spt-1(b104r1)*, nicknamed 'spadetail', was initially discovered among the parthenogenetic progeny⁵ of a

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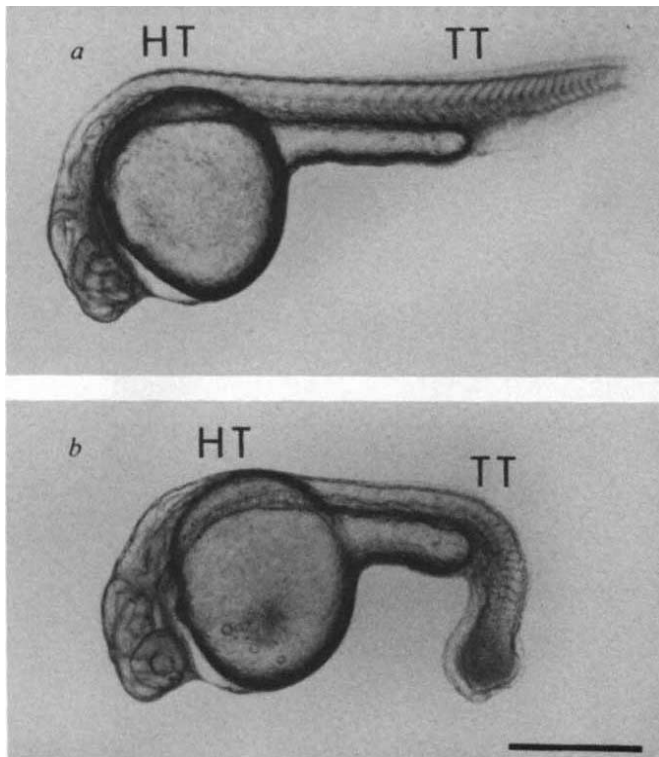


Fig. 1 Live wild-type (*a*) and homozygous *spt-1(b104)* mutant (*b*) zebrafish embryos at 24 h (all times are in hours postfertilization at 28.5 °C). Genetic nomenclature for zebrafish is adapted from that for *C. elegans*¹⁷; *spt-1* indicates the locus of the mutation, and *b104* is the allele. HT: region of transition between the head and trunk, at the level where the anterior-most somites are normally observed. These somites are missing in the mutant, and trunk paraxial (segmental plate) mesoderm is deficient. TT: region of transition of the trunk and tail, at the anus, that in the mutant marks the position of a severe kink of the body and the restoration of somites. The enlarged tail bud of the mutant contains cells that eventually degenerate. (The designation *spt* refers to this prominent phenotype, termed by us the 'spadetail'). Scale bar, 400 μ m.

single heterozygous female. The origin of the mutation is unknown. Mutants were identified by the changes in appearance of the embryo shown in Fig. 1. The phenotype is zygotic recessive, and mutants survive for less than one week of development. The mutation segregates as a single locus, and is fully penetrant; about one-quarter (410/1,660) of embryos obtained from crosses between heterozygotes were mutant, and about one-half (222/437) of haploid embryos obtained⁵ from heterozygous females were mutant. As a step towards characterizing the locus genetically, we have recently identified a putative allelic γ -ray-induced mutation, *b141*, by its failure to complement the original one, *b104*. We have not yet studied the phenotype of *b141* homozygotes however, and describe here the *b104* mutation only. We concentrate on the earlier changes, which are likely to be more useful for our eventual understanding of *spt-1*+ function.

Development seems normal before gastrulation, and during its onset. By six h (hours after fertilization at 28.5 °C) both wild-type embryos and *spt-1* mutants have begun epiboly⁶, and have formed a germ ring and embryonic shield⁷. The presence of these structures suggest that early morphogenetic cell movements occur in mutants, in particular the involution of mesoderm (ref. 8; R.M.W. and C.B.K., manuscript in preparation). After 7–8 h cells accumulate abnormally at the dorsal side of the gastrula to produce a transient swelling, whose anterior-posterior level corresponds approximately to the region of transition between the prospective head and trunk (HT; Fig. 2*a, b*). As epiboly is completed at 10 h, cells accumulate at a

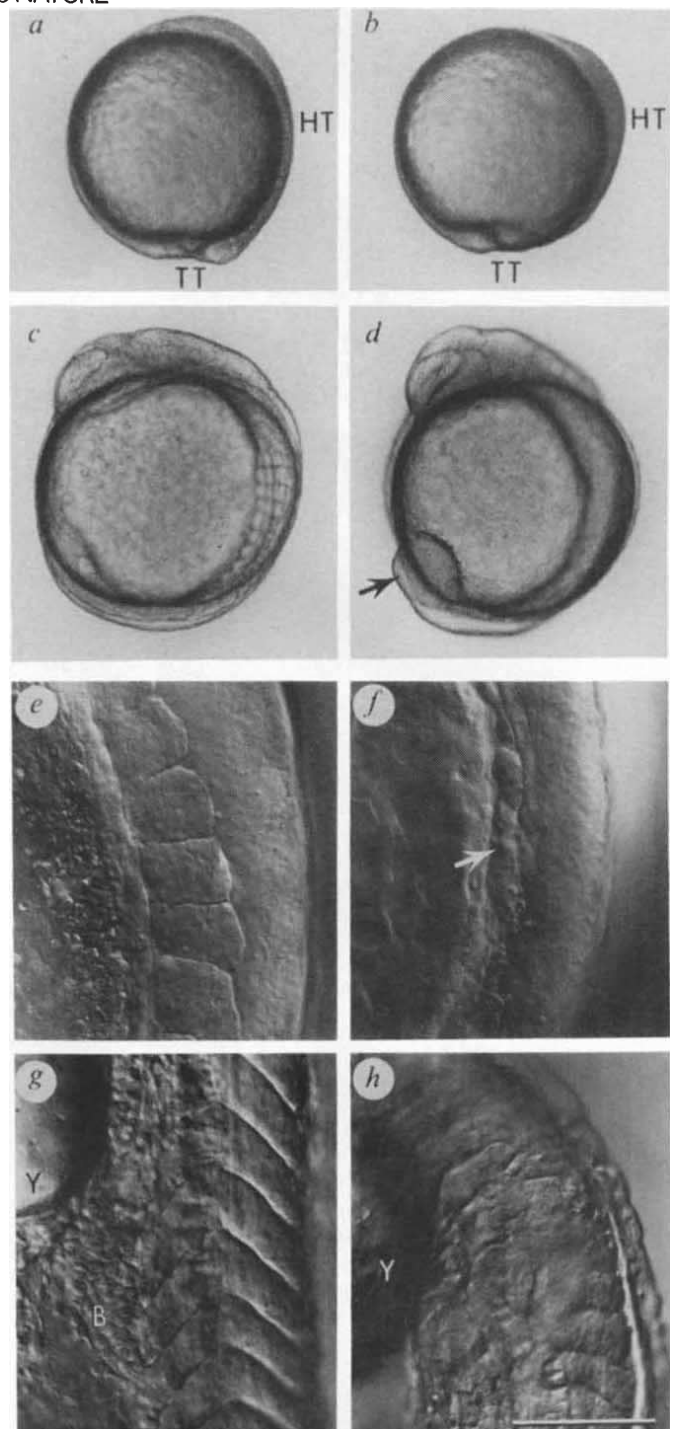


Fig. 2 Morphogenesis in living wild-type (left panels) and *spt-1* mutant embryos (right panels). Left side views with anterior to the top. *a, b*, Wild-types and mutants at completion of epiboly (closure of the yolk plug) at 10 h. The HT swelling was first observed in this mutant (*b*) at 7 h and the TT swelling has just appeared. *c, d*, Wild-type and mutant early tail bud embryos at 13.5 h. Trunk somites are visible in the wild-type (*c*), but are not present in the mutant (*d*), which by this stage has developed a very prominent tail bud (arrow). *e, f*, Nomarski interference contrast views of the developing trunk at 13.5 h. Somites are well-formed in the wild-type (*e*). The mutant (*f*) paraxial mesoderm (arrow) is very deficient; most of the tissue present is the spinal cord. *g, h*, Nomarski views of the TT region at 23 h. In the wild-type (*g*), V-shaped myotomes are present in both the trunk and tail. The anus (present but not visible at this plane of focus) is located just posterior to the yolk sac (Y), and near this location is a prominent cluster of developing blood cells (B). In the mutant (*h*), the first somites to appear are located over the posterior yolk sac. The myotomes that develop from them are poorly formed, whereas more posterior myotomes in the tail are better formed. The anus and blood cells are absent. Scale bars, 400 μ m (*a-d*); 100 μ m (*e-h*).

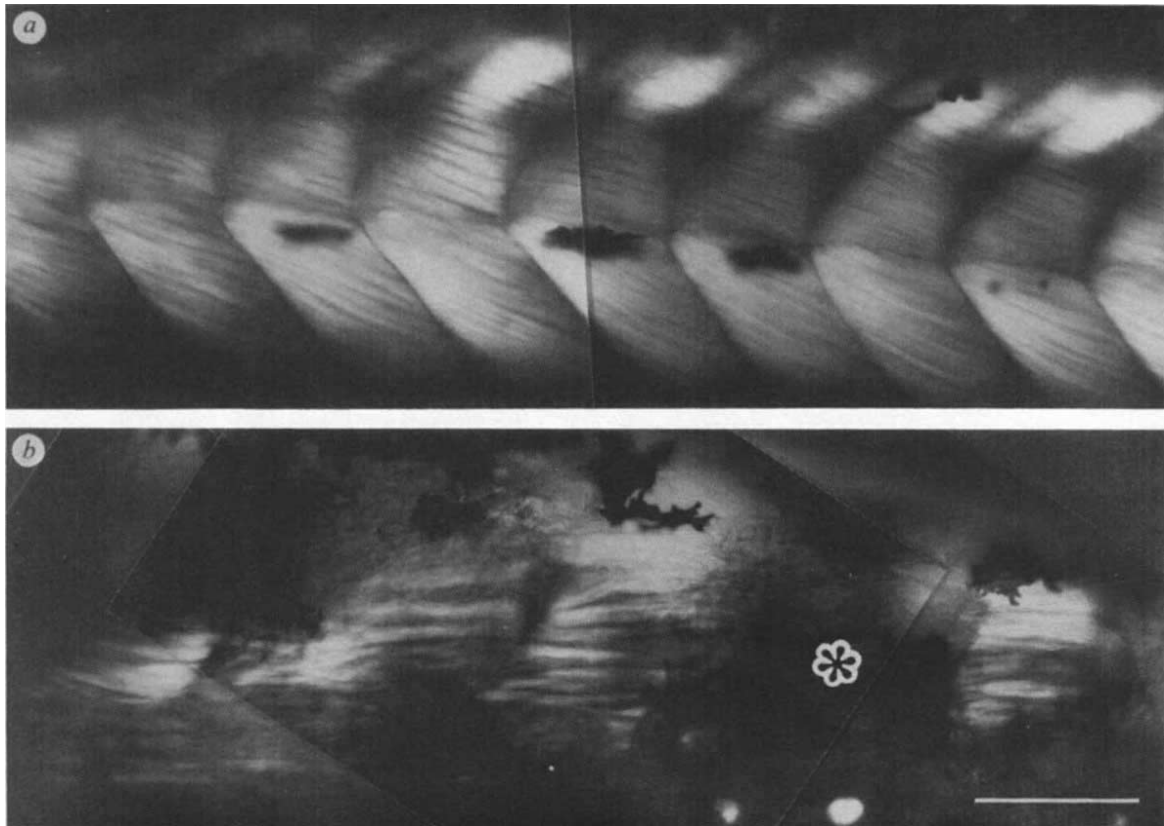


Fig. 3 Imperfect myotomes appear in the trunk in *spt-1* mutants. Left side views (dorsal to the top) of trunk segments in live embryos at 72 h. Polarized light, revealing patterning of birefringent myotomal muscle fibres. In wild-type embryos (*a*) the myotomes are regular V-shaped structures, and contain muscle fibres in orderly obliquely longitudinal orientations. The transverse myosepta (separating myotomes) and the horizontal myosepta (separating dorsal ventral parts of the myotomes) do not contain muscle, and are not birefringent. The view of a mutant (*b*) shows anterior trunk myotomes. The myotomes are of approximately normal length, and some muscle fibres are oriented longitudinally. Mutant trunk myotomes are not V-shaped, and horizontal myosepta are not detected. There are fewer muscle fibres than normal. Here, three anterior myotomes are followed by a myotome-long space with no muscle (*), and then by a single (isolated) myotome with severely disorganized fibres. Wild-type embryos normally have 15 myotomes anterior to the anus at this stage; this mutant had 6. Scale bar, 100 μ m.

second incorrect location, ventral to the location of yolk plug closure, and approximately at the region of the juncture of the trunk and the tail (TT; Fig. 2*a, b*). The cells in this second swelling then seem to enter the tail bud, which remains abnormally large as the tail develops (Fig. 2*d*, arrow).

During tail bud stages, paraxial mesoderm is depleted in the trunk region of *spt-1* embryos (Fig. 2*c-f*). As trunk somites are forming in wild-type embryos (11–16 h; Fig. 2*e*), there are only scattered mesenchymal cells, and a few clusters of cells adjacent to the notochord in the mutants (Fig. 2*f*, arrow). The notochord and overlying spinal cord of the trunk at first seem to be formed normally, and then develop bends and kinks, as if they were compressed along the anterior–posterior axis, with the most severe kinks near both the HT and TT. Later some mutants fail to develop structures that normally arise near the HT, including the pronephric kidneys and pectoral fins on one or both sides. Blood cells and the anus, both normally arising near the TT, may also be missing. Structures that lie anterior to the trunk are invariably present however, and develop fairly normally, for example, the brain (including hindbrain neuromeres⁹), head sense organs, the series of branchial arches, the heart, and the pericardial hatching gland.

At the posterior in *spt-1* mutants, beginning at about the TT, paraxial mesoderm is more abundant and somite furrows are observed, roughly on schedule (15–16 h) with the formation of tail somites in their wild-type sibs (Fig. 2*g, h*). The earliest somites to form in mutants, those near the TT, are poorly shaped at first, but the shape of these somites is better at later times,

and the posterior-most somites form rather normally at the outset. Eventually tail myotomes, derived from them, appear normal.

None of these changes are accompanied by an unusual amount of cell death. The only extensive cell death that we observe during embryogenesis in mutants is in the enlarged tail bud, beginning after the end of somitogenesis at about 30 h. First, a few necrotic cells can be identified in the tail bud with Nomarski optics¹⁰, and by 48 h degeneration is massive. As a consequence, the abnormally swollen tail bud (Fig. 1) eventually disappears. The cell death in the mutant tail bud might be an entirely normal morphogenetic process that is magnified by its excessive number of cells. We have observed some corresponding necrosis in wild-type embryos, and similar patterns of cell death have been observed after completion of segmentation in the chicken¹¹ and leech¹².

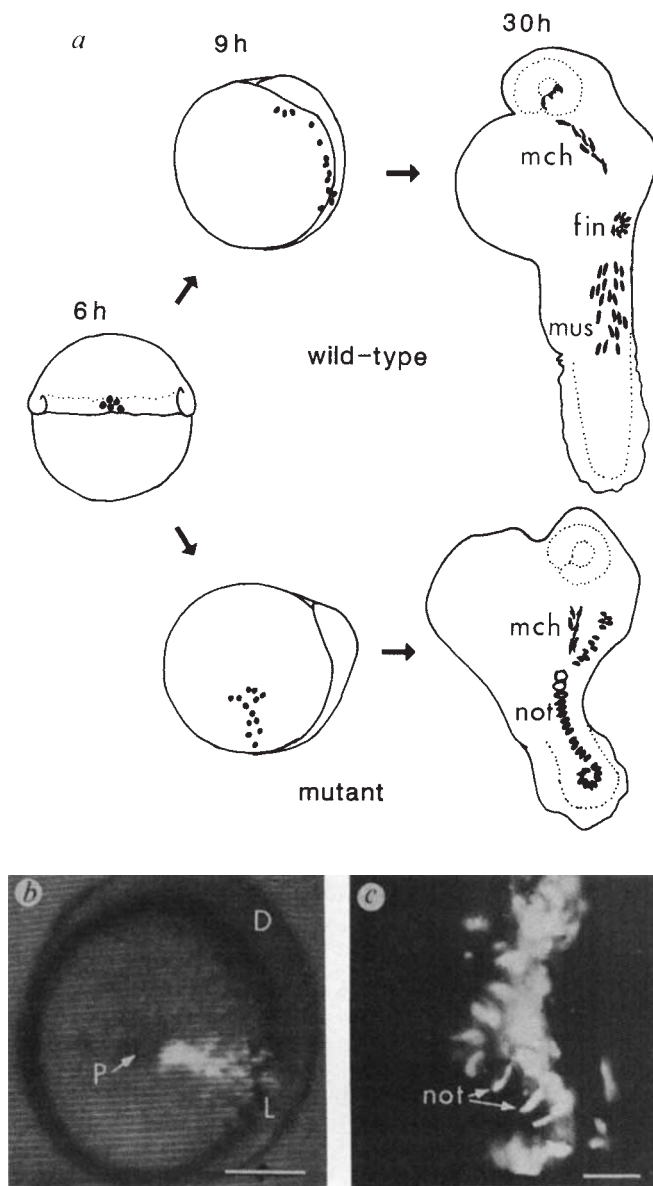
We interpret the phenotype to mean that the mutation most severely affects development of the trunk of the body. The trunk does not seem to be deleted altogether as, for example, in *Drosophila* gap mutants¹³. Some trunk structures, such as the notochord and the spinal cord, develop on a wild-type schedule. Moreover, a particularly interesting finding is that, later on, pattern regulation occurs in the trunk. Thus, before 24 h, myotomes become evident in this body region; that is, where no somites were present initially (Fig. 3). We have not determined the cellular origin of these myotomes. They are of about the correct axial length, but are not V-shaped as normal, and are sometimes separated from each other by myotome-long

Fig. 4 *a*, Cell movements and fates of prospective trunk somitic mesoderm are changed in *spt-1* mutants. Single clones of fluorescently labelled cells that occupied the lateral germ ring at 6 h (early gastrula) were followed in wild-type (top) and mutant (bottom) embryos. The drawings were made from video time-lapse sequences of images (as in *b*), and show left-side views with anterior to the top. In the wild type the cells converge dorsally by 9 h, and by 30 h have formed mesoderm of the head and trunk, including mesenchyme (mch), pectoral fin bud (fin) and somitic muscle (mus). In the mutant, the cells moved posteriorly to populate the caudal trunk and the tail. They formed mesenchyme, notochord (not) and mutant tail bud. The clones in both the wild type and mutant also included neural and surface epithelial derivatives, which are not shown in these drawings. *b*, A posterior view of this mutant at 9 h, photographed from the face of the video-monitor. Fluorescence due to the tracer is superimposed on the bright-field image. The labelled cells have formed a stream from their starting position in the lateral equatorial region (L) towards the yolk plug posteriorly (P). In wild-type embryos, cells at the same starting location move dorsally (D). *c*, Fluorescence video micrograph of the tail (side view) of the same mutant at 30 h. Differentiating notochord cells (not) are labelled, distinguishable by their position and distinctive disk-shape. The individual cells are separated from one another by unlabelled notochord cells, among which they have intercalated earlier in development; the intercalations are a normal feature of morphogenesis¹⁵. In wild-type diploid embryos (as in *Xenopus laevis*¹⁸) the notochord of the head, trunk, and tail derives from cells located adjacent to the dorsal blastoderm margin of the early gastrula (C.B.K. and R.M.W., manuscript in preparation). In the work presented here we observed that dorsal marginal cells also gave rise to notochord in both mutant and wild-type haploid embryos (three out of five clones in mutants and three of 10 clones in wild types; at the gastrula stage these clones were all located along the dorsal one-third of the germ ring). Lateral and ventral marginal cells frequently gave rise to notochord in haploid mutants (five of 12 clones that were located along the lateral and ventral two-thirds of the germ ring in the gastrula), but not in wild types (none out of seven lateral and ventral clones). A more complete description of the mutant fate map is in preparation.

Methods. As mutants could not be identified until after the beginning of these experiments, we obtained a high proportion (50%) of mutants, by using haploid progeny⁵ from *spt-1/+* heterozygotes. The mutant phenotype in haploids is similar to, and generally more severe than, that of homozygous diploids. Single clones were marked in a series of embryos at about 3 h (about the 10th cleavage division), by injecting cells near the blastoderm margin with rhodamine-dextran¹⁹. The position of the clones was mapped at early gastrula stage (6 h) using a Silicon-Intensified-Target (SIT) video camera to amplify fluorescence. In some cases of embryos bearing lateral clones (positioned at 90° from the embryonic shield⁷) the movements of the labelled cells were recorded during the next nine hours by time-lapse video microscopy. At 30 h we determined whether the embryos were wild-type or mutant, and scored the positions and the differentiated fates of the labelled cells⁷. Scale bars, 200 μ m (*b*); 50 μ m (*c*).

spaces that contain no muscle (Fig. 3*b*, *). The correct number of myotomes (30 pairs, including the trunk and tail) is also not restored in *spt-1* mutants. Counts in mutants range upwards from about 14.

Many of the defects in *spt-1* mutants may arise secondarily, cascading from an early pattern disturbance that involves the movements of specific cells during gastrulation. The HT and TT mark both the early locations of the abnormal cell accumulations and the later regions of most severe pattern defects. Paraxial mesoderm (prospective somite) is deficient between these same two regions (that is, in the trunk). These results indicated that those cells which should have migrated in the gastrula to form trunk paraxial mesoderm, moved incorrectly, leaving the trunk cell-deficient. We examined this possibility directly by using intracellularly injected tracer dye to observe the movements and eventual fates of the mutant cells. Previous work^{7,14} established that the trunk somitic mesoderm normally arises by the dorsal-wards convergence of cells from the lateral part of the germ ring at early gastrula stage. These cell movements seem to be similar in the haploid wild-type embryos studied here, and cell fates include trunk somitic muscle (Fig. 4*a*, top). However, in haploid *spt-1* mutants (Fig. 4*a*, bottom) cells at the same location in the early gastrula fail to converge dorsally; rather they move



posteriorly, in an apparently active fashion (Fig. 4*b*), to enter the tail. The derivatives of such dislocated cells often include unusual fates for cells from this beginning location, in particular the notochord (Fig. 4*c*). Notochord of both the trunk and tail is normally derived from the dorsal side of the early gastrula, not the lateral side (see Fig. 4 legend). Thus the *spt-1* mutation can change the fate, as well as the movements of mesodermal cells. On the other hand, transfating was not invariably observed; in some mutants the dislocated cells formed tail muscle.

From the early expression of the mutant phenotype we infer that *spt-1* must normally function during (or before) gastrulation. Our studies have not revealed the primary gene function, or which cells express the gene. The earliest observed change is in cell movement, and perhaps the mutation directly perturbs movement—affecting either the mesodermal cells themselves or their environmental navigation cues. If so, the observed changes in pattern and cell fate could follow secondarily, because final cell positions are changed. Alternatively, the mutation could act more cryptically: if cell commitments occur during gastrulation¹⁵, the mutation could alter those commitments, and the cells move improperly as a consequence. Study of embryos that are genetically mosaic¹⁶ for *spt-1*, as well as determining the primary function of the gene, should elucidate our

understanding of the relationships of cell movement, position and fate during embryogenesis in this vertebrate.

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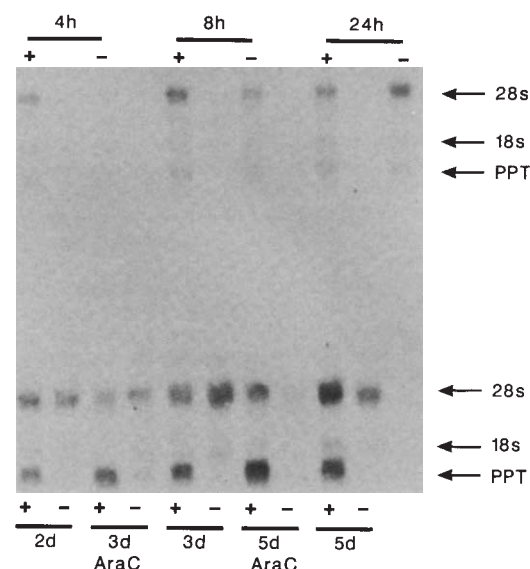


Fig. 1 Northern blot analysis of the effect of NGF upon the levels of PPT mRNA in adult rat DRG neurons. Replicate cultures of enriched adult DRG neurons (10,000 neurons per well) were established at $t = 0$. The mitotic inhibitor cytosine arabinoside (Ara C, 10^{-5} M) was present in replicates of the 3-day and 5-day cultures. At each time point, the neurons from four wells, grown in the presence or absence of NGF, were pooled, and total RNA was isolated. RNA samples were electrophoresed on formaldehyde-agarose gels and probed with a 32 P-labelled RNA probe complementary to human PPT mRNA. Under the washing conditions used ($2 \times$ SSPE/0.1% SDS at 65°C), the autoradiographic signals due to the nonspecific binding of probe to 28S and 18S RNAs could be used to compare the amounts of RNA on each lane of the gel. Under more stringent conditions ($0.1 \times$ SSPE at 65°C) the intensity of the signal due to PPT mRNA was undiminished, but the nonspecific binding of probe to RNA was abolished.

Methods. Cultures of adult rat DRG neurons were prepared as previously described². Briefly, pooled DRG (40-48 per animal) from adult Sprague-Dawley rats (200-250 g) were cleaned of connective tissue and dissociated into a suspension of single cells by trituration after collagenase and trypsin treatment. After enrichment by separation of neurons from non-neuronal cells on the basis of their differential adhesion to a polyornithine substrate after overnight incubation, 10,000 neurons were plated out in each of the four wells (coated with polyornithine and laminin) of 35 mm-well tissue culture dishes (Greiner). The cells were maintained at 36.5°C , in an atmosphere of 97% air: 3% CO_2 in F-14 culture medium (Imperial) supplemented with 10% horse serum (GIBCO-BRL), and, where indicated, cytosine arabinoside (10^{-5} M; Sigma). Highly purified 2.5 S mouse salivary gland NGF was added at 25 ng ml^{-1} . The medium was changed after 3 days where appropriate. For Northern analysis, RNA samples, each obtained from 40,000 neurons, were electrophoresed on 1.1% agarose gels containing formaldehyde, and transferred to nylon membranes (Biohyne A: Pall Ultrafine Filtration, Glen Cove, New York). A radiolabelled β -PPT cRNA probe was obtained by transcription, using [α - 32 P]CTP and T7 RNA polymerase, of a fragment of human β -PPT cDNA (bases 174-417 of the published sequence¹⁸ which had been subcloned into the Gem3 vector (Promega). The human cRNA probe was more than 95% homologous to the corresponding region of rat β -PPT mRNA¹⁹.

Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons

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Nerve growth factor (NGF) is a trophic molecule essential for the survival of sympathetic and sensory neurons during ontogeny¹. The extent to which NGF is involved in the maintenance or regulation of the differentiated phenotypes of mature peripheral neurons is much less clear, however. Biochemical analysis of the actions of NGF upon peripheral neurons has been hampered by the lack of a preparation of neuronal cells that are responsive to NGF but do not require it for survival. We report here that in adult dorsal root ganglion neurons, which can be isolated, enriched and maintained in culture in the absence of neuronal growth factors², the expression of mRNAs encoding the precursors of two neuropeptides, substance P and calcitonin gene-related peptide is regulated by NGF. Our results provide the first direct evidence of a continuous dynamic role for NGF in regulation of peptide neurotransmitter/neuromodulator levels in mature sensory neurons.

When adult dorsal root ganglion (DRG) neurons were grown in culture in the absence of NGF, the level of mRNA encoding the substance P precursor (preprotachykinin, PPT) declined progressively over 5 days (Figs 1, 2a). The substance P content of parallel cultures (Fig. 2b) declined by 60-70% over the same period. In cultures maintained in the presence of NGF (25 ng ml^{-1}), levels of PPT mRNA did not differ significantly from controls for the first 24 h, rose 1.5-4-fold (range in six experiments) over the next 24-48 h and did not change significantly thereafter. The abundance of PPT mRNA was 4-6-fold greater in cultures exposed to NGF for 48 h, compared with

those deprived of NGF. After 5 days, PPT mRNA levels were 15-60-fold higher in NGF-treated cultures than in untreated cultures, as much because of a decline in PPT mRNA in NGF-deprived cultures as a continuing increase in PPT mRNA levels in response to NGF. Over the time course of these studies, the substance P content of NGF-treated cultures rose by approximately 50%.

Most, if not all, of the subpopulation of rat DRG neurons which exhibit substance P-like immunoreactivity also contain

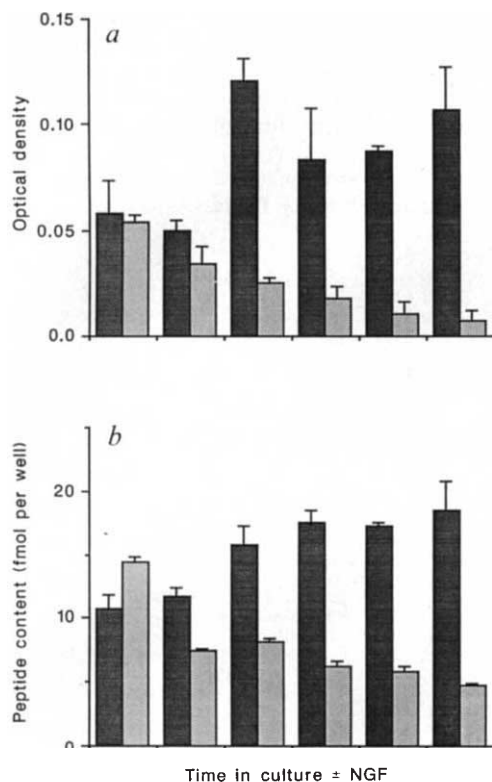


Fig. 2 Effect of NGF upon levels of (a) PPT mRNA and (b) substance P-like immunoreactivity in replicate cultures of adult rat DRG neurons maintained for the times shown in the presence (dark blocks) or absence (pale blocks) of NGF (25 ng ml⁻¹).

Methods. a, Northern blots of total cellular RNA (derived from 40,000 neurons per sample) were hybridized with a PPT cRNA probe (see Fig. 1 legend) and results quantitated by densitometry using a Cambridge Instruments Quantimet 800 image analyser. b, The substance P-like immunoreactivity content of parallel cultures (5,000 neurons per sample) was determined by radioimmunoassay using a C-terminally directed, polyclonal substance P antiserum²⁰. Values are mean ± s.e.m. (n = 4).

calcitonin gene-related peptide (CGRP)³. To establish whether the effects of NGF we described are specific for substance P or also affect the expression of other neuropeptides, we examined the levels of CGRP and its mRNA in the same or parallel cultures to those used above. As for substance P, the levels of CGRP mRNA declined (Fig. 3) in cultures deprived of NGF. CGRP mRNA levels were more than 15-fold higher in cultures treated with NGF for 5 days, than in similar cultures deprived of NGF. In parallel cultures in which CGRP content was measured by radioimmunoassay, a 4-fold increase in CGRP was detected over 4 days in culture in the presence of NGF (data not shown).

Although we have previously shown that the vast majority of adult rat DRG neurons survive in culture in the absence of NGF or other neurotrophic factors², we had to establish that the effects described above were not due to NGF promoting the survival of a small population of substance P-rich DRG neurons. Cultures were first deprived of NGF for 2 days (in total 3 days without NGF, including the enrichment procedure) and NGF was then added for 2 or 4 days, RNA was isolated, and PPT mRNA measured. Figure 4 shows that levels of PPT mRNA were elevated in NGF-treated cultures regardless of prior deprivation of NGF. We have shown previously that, even after NGF deprivation for 6 or more days, substance P levels in cultured adult DRG neurons can be elevated upon the addition of NGF⁴. We conclude, therefore, that the effect of NGF on PPT and CGRP mRNAs is not linked to survival but is due to a separate action of NGF on the function of adult sensory neurons.

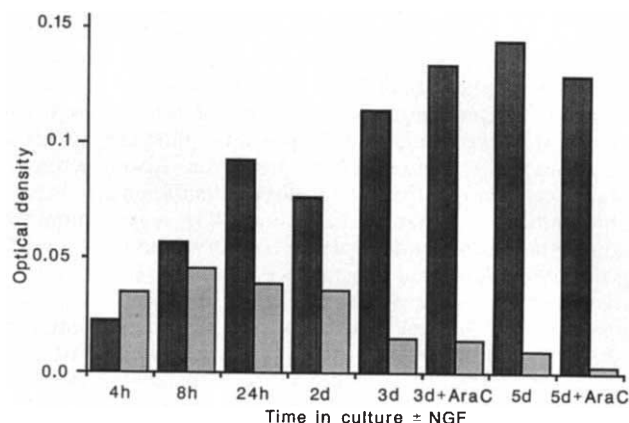


Fig. 3 Effect of NGF on the level of CGRP mRNA in duplicate cultures of adult rat DRG neurons maintained in the presence (dark blocks) or absence (pale blocks) of NGF (25 ng ml⁻¹) and, where shown, cytosine arabinoside (Ara C: 10⁻⁵ M). Northern blots of total cellular RNA (see Fig. 1 legend) were probed with a CGRP cRNA probe and the results quantitated by densitometry. The probe used was complementary to a 230-base pair sequence from 3'-untranslated region of human α -CGRP mRNA (ref. 21), ~76% homologous to the corresponding region of rat α -CGRP mRNA (ref. 22), and was generated using a commercial kit (Amersham).

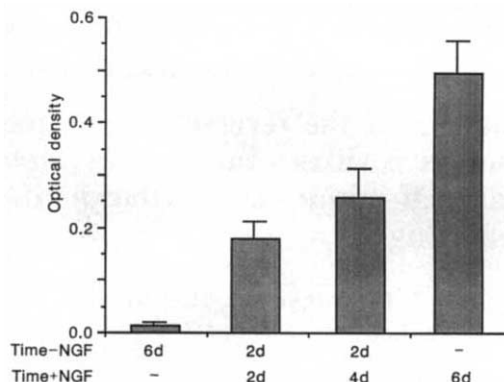


Fig. 4 Comparison of the relative levels of PPT RNA in replicate cultures of adult DRG neurons that were grown continuously in the presence of NGF, without NGF or in which the addition of NGF was delayed for 2 days after plating. Note that PPT mRNA levels were elevated (compared to controls) even after initial deprivation of NGF for 2 days. Values are mean ± s.e.m. (n = 3).

Although the procedures used to establish adult rat DRG neuronal cultures give an initial enrichment of >80% neurons, the proliferation of non-neuronal cells (Schwann cells and fibroblasts) cannot be suppressed without mitotic inhibitors such as cytosine arabinoside. To show that non-neuronal cells did not contribute directly or indirectly to the effects observed, several additional experiments were carried out. First, no PPT or CGRP mRNA was detectable in enriched cultures of non-neuronal cells grown in the presence or absence of NGF. Second, although cytosine arabinoside strongly inhibited the proliferation of non-neuronal cells in culture, this mitotic inhibitor did not influence the regulation of PPT and CGRP mRNA by NGF (Figs 1, 3).

There is now strong evidence that DRG neurons, once mature, do not depend on NGF for survival, as they do during development^{5,6}. Instead, several lines of indirect evidence suggest that, in adult animals, NGF may be important in regulating the production of neuropeptides in sensory neurons. Various chemical, immunological and surgical interventions *in vivo* have been shown to lead to reductions in the levels of substance P

in adult DRG. For example, sciatic nerve section in the rat results in the loss of substance P from lumbar DRG, an effect attributed to NGF deprivation, as the substance P loss can be reversed by application of NGF to the cut stump of the transected nerve⁷. Given the considerable cell death in DRG after peripheral nerve section, it is possible⁸ that the effects of exogenous NGF are related to protection against axotomy-induced cell death, rather than to direct stimulation of substance P biosynthesis. Further evidence that NGF may dynamically regulate the neuropeptide content of sensory neurons is provided by the observation that substance P levels are elevated in the target fields of sensory neurons after chemical (6-hydroxydopamine) or surgical destruction of NGF-dependent sympathetic neurons innervating the same structures; this elevation of substance P levels is probably due to an increased availability of target-derived NGF for the intact sensory neuron^{9,10}.

The observed differences in the levels of substance P or other peptides after NGF deprivation or administration *in vivo* are not evidence that NGF modulates the genes encoding the precursors of these peptides. Increased or decreased peptide levels could simply be a consequence of changes in the rates of translation, processing, release or degradation of the peptides themselves. The observed actions, the direct induction of the mRNAs, were not mediated indirectly through non-neuronal cells, nor were they linked to a survival-promoting effect of NGF. Our results suggest that a continuous supply of target-derived NGF is required as a retrograde effector to maintain the steady state levels of peptide neurotransmitters/neuromodulators found in mature sensory neurons. NGF treatment has

been shown to evoke a number of rapid biochemical changes (including the phosphorylation of specific proteins¹¹⁻¹³ and the induction of certain genes¹⁴⁻¹⁷) in PC12 pheochromocytoma cells. The system used here provides a means to establish the relevance of these observations to the mechanism of action of NGF in normal cells.

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Biosynthesis of the reverse transcriptase of hepatitis B viruses involves *de novo* translational initiation not ribosomal frameshifting

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Retroviruses and many other types of genetic elements replicate by reverse transcription of RNA (for reviews, see refs 1 and 2). Although structurally and biologically very diverse, such elements carry conserved polymerase genes (*pol*) that encode proteins required for reverse transcription³. In most cases, the *pol* gene is preceded by an overlapping gene encoding one or more nucleocapsid proteins⁴, in a different reading frame. Because both coding regions are represented in a single mRNA, the question arises of how the reverse transcriptase in the alternative reading frame is expressed. In retroviruses and retrotransposons it is expressed as a nucleocapsid-polymerase fusion protein by ribosomal frameshifting during translation of the overlapping region⁵⁻⁸. We have examined the mechanism of polymerase biosynthesis in another family of animal viruses that use reverse transcription, the hepatitis B viruses. Genetic and biochemical studies reveal that these viruses do not use ribosomal frameshifting to generate this enzyme, but instead direct translation initiation at an internal initiation (AUG) codon in the polymerase gene.

The hepatitis B viruses (HBVs) are small DNA viruses that produce persistent hepatic infections in a variety of animal hosts and replicate their DNA genomes via reverse transcription of an RNA intermediate⁹. All members of this family contain an open reading frame (ORF), "P" (for *pol*), which is homologous

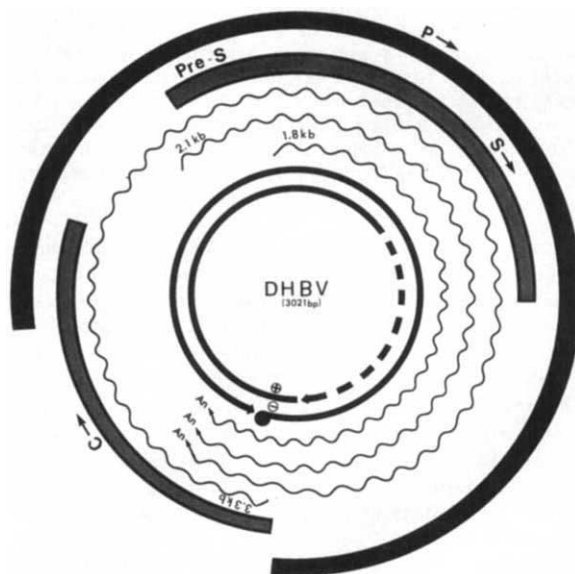


Fig. 1 Physical map of DHBV. Inner circles represent the DNA genome: the minus (—) strand (the strand synthesized through reverse transcription of viral RNA) is linked to a terminal protein³⁵ shown as a solid circle at its 5' end; the plus strand is partially incomplete and shown by a dashed line at the 3' end. The three wavy lines are the three main species of viral transcripts found in infected cells: the 2.1 kilobase (kb) mRNAs coding for the pre-surface (pre-S) antigens, the 1.8 kb mRNAs coding for the surface (S) antigens, and the 3.3 kb RNAs being the viral pregenome (the template for reverse transcriptase) and mRNAs coding for the core (C) antigens and viral polymerase (P). The three long open reading frames for S, C and P (*pol*) genes are drawn as filled boxes. Note that the 5' end of the P gene is overlapped by the 3' end of the C gene. For detail see ref. 36.

to retroviral *pol* genes. As Fig. 1 shows for the duck hepatitis B virus (DHBV)¹⁰, the 5' end of the P frame overlaps with the coding region (C) for the core (or nucleocapsid) protein; *pol* sequences are in the +1 frame relative to *core*. Figure 1 also shows the principal mRNAs identified in infected livers¹¹⁻¹⁴. These include the subgenomic RNA species encoding the viral surface (S) proteins and the genome-length RNAs, one of which serves as the template for reverse transcription. As for other retroviral elements, only the genome-length RNA contains the entire *pol* region. Such RNA is found in polyribosomes and is believed to be the mRNA for translation of both *core* and *pol* proteins. As no other (for example, spliced) viral RNAs have been reported in hepadnavirus infected cells, the main possibilities for polymerase biosynthesis are translational frame-shifting to produce a *core-pol* polypeptide or translational initiation at an AUG in the *pol* gene. To date, no intact *core-pol* fusion proteins have been detected in productively infected cells (although truncated *core-pol* fusions were detected in some tumours in which viral DNA was probably rearranged¹⁵). However, *pol*-related proteins of relative molecular mass 70,000–90,000 (70–90K), without associated *core* determinants, have been identified in HBV virions^{16,17}. It is unclear whether these polypeptides are the primary translation product of the *pol* gene or result from proteolytic cleavage of a large *core-pol* precursor.

To study *pol* biosynthesis we initially used a strategy which had been useful in the demonstration of translational frameshifting in retroviruses⁵. The *core-pol* overlap of ground squirrel hepatitis virus was cloned downstream of an SP6 bacteriophage promoter and *in vitro* transcripts were translated in extracts of rabbit reticulocytes, wheat germ, mouse liver and infected ground squirrel liver in attempts to identify a frameshift product. In all experiments, a detectable but low level ($\leq 1\%$) of translational frameshifting occurred within the *core-pol* overlap (data not shown). Control experiments using the *pol-S* overlap (see Fig. 1) also exhibited this low level of frameshifting, suggesting that little or no specific frameshifting was occurring in the *core-pol* overlap region.

To assess the importance of the *core-pol* overlap *in vivo*, we constructed mutations in the *core* and *pol* genes designed to identify regions of the genome required for *pol* biosynthesis, using the DHBV genome. DHBV DNA can initiate infection of either intact animals (when delivered by intrahepatic inoculation¹⁸⁻²⁰) or of cultured human hepatoma cell lines^{21,22} (following transfection with cloned viral DNA^{23,24}). For the assay in animals, one-day-old ducklings were intrahepatically injected with mutant DNA, and 18–21 days later (to allow for horizontal spread of progeny virus) viral replication was scored by Southern blot analysis of liver DNA. In the cell culture assay, 3–5 days post-transfection, viral core particles were purified by density centrifugation or immunoprecipitation²⁵, and DNA extracted from the cores was analysed by blot hybridization. Authentic DHBV replication generates a characteristic array of DNA forms, including open circular and linear duplexes and a heterogeneous array of linear single-stranded and partially duplex circular forms (see, for examples, Fig. 2b, lanes 2 and 4). Site-specific mutations were generated using synthetic oligonucleotides²⁶. Whenever possible, changes in *pol* were made so as not to affect *core*.

In this strain of DHBV, the *core-pol* overlap is 305 nucleotides (nt) with two AUG codons in the *pol* frame; the A residue of the first AUG is designated position 1 (Fig. 2a). Note that the *pol* frame is open for 60 nt 5' to its first AUG. We first constructed a frameshift mutant in a region near the 3' end of the *core-pol* overlap (mutant 253, Fig. 2a) by deletion of a single nucleotide at that position, which places a termination codon in the *pol* frame 10 nt downstream of the mutation. None of the four ducks inoculated with 253 DNA became infected, and no viral DNA synthesis was observed after introduction of 253 DNA into cultured cells (Fig. 2c). This indicates that sequences upstream of this site are required for production of *pol* gene products.

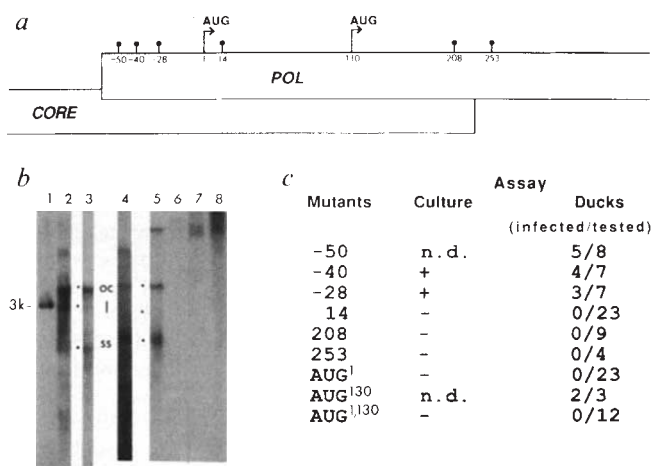


Fig. 2 Effects of mutations in the *pol* frame. *a*, Schematic representation of the *core* and *pol* overlap. Open boxes represent ORFs. In the overlap, the two AUG codons found in *pol* are marked by arrows. Individual nonsense mutations were made at the positions indicated by the closed circles (except mutant 253 which is a frameshift mutant). Each mutant contains sequence changes in both *core* and *pol* as follows: mutant 253, GCT (Ala) to GC in *pol*; mutant 208, CGA (Arg) to TGA (opal) in *pol* and TCG (Ser) to TTG (Leu) in *core*; mutant 14, TTG (Leu) to TAG (amber) in *pol* and ATT (Ile) to ATA (Ile) in *core*; mutant -28, TATca (Tyr) to TAAgc (ochre) in *pol*, and TCA (Ser) to AGC (Ser) in *core*; mutant -30, TGCgt (Cys) to TGAga (opal) in *pol* and CGT (Arg) to AGA (Arg) in *core*; mutant -40, TTTct (Phe) to TAAgt (ochre) in *pol* and TCT (Ser) to AGT (Ser) in *core*; mutant AUG¹, AUG (Met) to ACG (Thr) in *pol* and GAT (Asp) to GAC (Asp) in *core*; mutant AUG¹³⁰, AUG to ACG in *pol* and TAT (Tyr) to TAC (Tyr) in *core*. *b*, Southern blot of the replicative forms of viral DNA synthesized in duck livers or tissue culture following transfection. A DHBV probe was used to detect viral-specific sequences. Liver DNA samples from ducks tested with the wild-type DHBV DNA (lane 2) and the mutant -28 DNA (lane 3), and mutant -28 DNA from cytoplasmic cores extracted from transfected Huh7 cells (lane 4) are shown. DNA from cores prepared from HepG2 cells transfected with DNA of wild-type (lane 5), mutant 14 (lane 6), AUG¹ mutant (lane 7) and AUG^{1,130} double mutant (lane 8) are also shown. Three major replicative forms of viral DNA are indicated by black dots; 'oc', open circular; 'l', linear; 'ss', single stranded. A linear 3 kbp DHBV DNA marker is shown in lane 1. *c*, Summary of DHBV mutants assayed both in tissue culture and in ducks. The results of duck inoculation experiments are shown to the right. '+' and '-' indicate positive and negative for *pol* function in HepG2 and/or Huh7 cells respectively. n.d., Not determined. The AUG¹ and AUG^{1,130} mutants and mutant 14 all expressed a low level of *pol* activity detectable only after a long exposure of the autoradiograms; however, this activity does not support detectable viral replication in ducks.

To define further the boundaries of sequences required for *pol* expression, we analysed the behaviour of mutants with lesions in the *core-pol* overlap. To explore the 5' boundary, several nonsense mutations were introduced in the *pol* ORF 5' to its first AUG. Three of these, at positions -50, -40 and -28 relative to this AUG, do not affect the sequence of the *core* product, and all are viable in ducks and cultured cells (Fig. 2). Representative data for mutant -28 are shown in Fig. 2b, lanes 3 and 4. These data indicate that translation of *pol* must begin downstream of position -28. To map the 3' boundary, nonsense mutations at positions 14 and 208 of *pol* were similarly analysed. As summarized in Fig. 2c, neither mutant grew in ducks and neither produced replicative intermediates in culture (see Fig. 2b, lane 6 for mutant 14). Therefore, sequences critical for *pol* expression are localized within a 42-nt region from -28 to +14 in the *core-pol* overlap. As this region contains an AUG codon, the contribution of this AUG to the generation of polymerase activity was addressed by further mutational studies.

If an internal initiation model is correct, the above results indicate that expression of the *pol* gene probably depends on the first of the two AUGs. When we changed the second AUG (AUG 130) to an ACG codon (without affecting *core*), two of three ducks tested positive for viral growth (Fig. 2c, mutant AUG¹³⁰), indicating that the second AUG is dispensable for *pol* function. However, when the first AUG of the *pol* ORF was mutated to an ACG codon (again without affecting *core*), the mutant did not replicate either in culture or in animals (Fig. 2b, lane 7, and Fig. 2c). Furthermore, a DHBV mutant with both of the AUG codons changed to ACG also failed to replicate in ducks (Fig. 2c, mutant AUG^{1,130}) or in culture (Fig. 2b, lane 8). These data suggest that the first AUG codon in the 42 bp region defined above is critical for viral *pol* expression or function. These results do not, however, exclude the possibility of ribosomal frameshifting within a small window from -28 to the first AUG, if this methionine was critical for *pol* function.

To define further the sequences in the *core-pol* overlap required for synthesis of *pol* protein, we constructed two amber mutants in the *pol* ORF at position -9 and -3 relative to the first AUG codon. When assayed, neither mutant replicated in ducks or in tissue culture (Fig. 3a). But as both contain a missense change in the *core* gene as well, the effects of the amber mutations on *pol* expression are ambiguous. A complementation assay was therefore developed to test the polymerase function independently of *core* function in tissue culture. A DHBV clone, DpA1, was constructed with multiple *pol* mutations, including the two AUG changes (at positions 1 and 130) described above and a 395-bp insertion downstream, yet maintaining the *core* amino-acid sequences intact (see Fig. 3b). This mutant expressed normal levels of viral mRNAs and *core* proteins (data not shown). As expected, the mutant is *pol*⁻: when transfected alone into cells, DpA1 DNA produced no viral replicative intermediates (Fig. 3c, lane 2). The same result was observed for the -3 amber *pol* mutant (Fig. 3c, lane 3), but when this mutant DNA was cotransfected with DpA1, replicative forms of viral DNA were detected (Fig. 3c, lane 4). Similar results were obtained with the -9 amber mutant (data not shown).

It is very unlikely that DNA recombination can cause this result. No replicative forms were seen in control cotransfection experiments using two *pol* mutants, that is, DpA1 plus *pol* mutant 253 or 208 (data not shown). In addition, to generate wild-type DNA from the -3 *pol* mutant and DpA1, recombination must occur within 2 bp (see Fig. 3b). To exclude DNA-DNA recombination further, however, we conducted an RNA-DNA cotransfection experiment. RNA containing only *core* sequences was synthesized *in vitro* using SP6 RNA polymerase and introduced into cells together with DNA carrying either the -9 or the -3 *pol* lesions. The products of viral polymerase activity were present in cell extracts collected 48 h post-transfection (Fig. 3d).

Finally, we investigated whether *pol* production is dependent upon translation of the antecedent *core* gene, a strong prediction of the frameshifting model. We constructed the plasmid Dfs1, which has a frameshift mutation in the *core* ORF, terminating *core* translation well upstream of the *core-pol* overlap region (Fig. 4a). As expected, this mutant is not replication-competent in culture (Fig. 4b, left panel, lane 2). The ability of this mutant to generate a functional *pol* product was then tested using complementation assays. When this clone was cotransfected with DNA of the known *pol*⁻ mutant DpA1 (Fig. 3b), viral replication intermediates were produced (Fig. 4b, left panel, lane 1), indicating that Dfs1 does indeed produce *pol* proteins.

The possibility of DNA-DNA recombination was excluded by showing that the defect in Dfs1 could be complemented by the provision of the *core* function alone in an RNA-DNA cotransfection experiment. As shown in Fig. 4b (right panel, lane 1), when Dfs1 DNA was cotransfected with *core* mRNA, viral replicative intermediates were produced. As a control, cotransfection of Dfs1 with the SP6-*core* DNA template (which

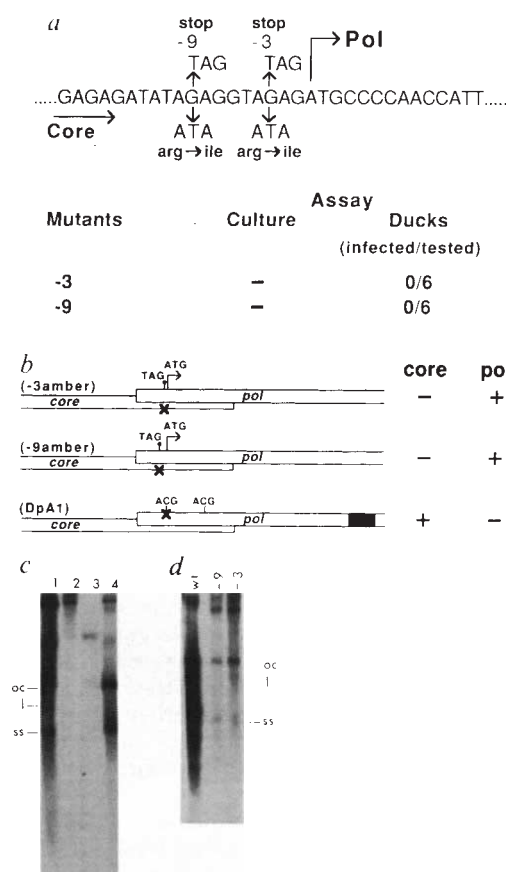


Fig. 3 Assay for *pol* function of two *pol* amber mutants immediately 5' to the first *pol* AUG. *a*, Assays in animals and cell culture. Point mutations were created individually at positions -3 and -9 in the *core-pol* overlap. As shown in the sequence both mutants create simultaneously a stop codon in the *pol* frame (arrow directed up) and a missense mutation in the *core* frame (arrow down). *b*, Schematic structure of the -3 and -9 *pol* amber mutants and the DpA1 clone. Both amber mutants bear a missense lesion in *core* (leading to a nonfunctional *core* protein) indicated by the 'x'. The DpA1 bears two lesions in *pol* that affect the phenotype, one at the first AUG codon (the 'x') and the other an insertion in the middle of *pol* (the filled box). The second AUG to ACG mutation at +130 does not affect viral function (Fig. 2). The phenotypes determined by tissue culture assay are shown to the right. *c*, Complementation assay by DNA-DNA cotransfection. Lane 1, wild-type DNA alone; lane 2, DpA1 DNA alone; lane 3, the -3 amber mutant DNA alone; lane 4, DpA1 plus -3 amber mutant DNA. *d*, Complementation assay by RNA-DNA cotransfection. 'wt', Wild-type DNA alone; '-9', SP6 *core* RNA and -9 amber mutant DNA; '-3', SP6 *core* RNA and -3 amber mutant DNA.

Methods. For the construction of DpA1, a 395 bp fragment from *Staphylococcus aureus* protein A gene was cloned into the *pol* ORF at the *Kpn*I site (near the *S* gene AUG) using the AUG^{1,130} mutant clone. DNA-DNA cotransfection was performed by the use of same amount of both DNAs (15 µg each in a 100-mm dish). The RNA-DNA cotransfection used poly-L-ornithine³⁷ at 20 µg ml⁻¹ and only HepG2 cells were used for this assay.

does not contain the DHBV promoter), did not allow the production of *pol* (the right panel, lane 2); thus any homologous recombination between incoming DNA molecules is not sufficient to allow detectable marker-rescue of the *core* frameshift mutation. These results indicate that *pol* expression does not depend on translation of *core*.

We have presented several lines of evidence to indicate that the hepadnaviral reverse transcriptases are not made as *core-pol* fusion proteins by ribosomal frameshifting. Hepatitis B viruses thus differ from all well-studied retroviruses and retrotrans-

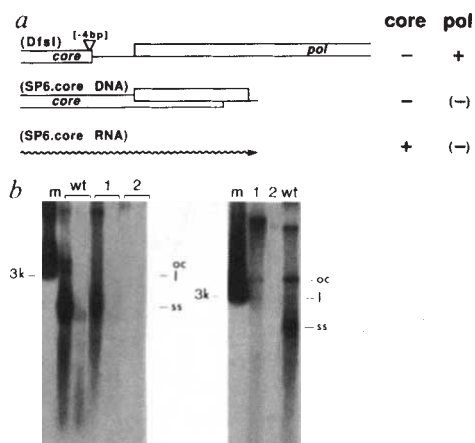


Fig. 4 Complementation assay with the *core* frameshift mutant Dfs1. **a**, Schematic structure of the Dfs1, SP6 core DNA and SP6 core RNA. In Dfs1, fill-in of the unique *Nsi*I site in *core* causes frameshift and premature termination (two codons downstream of the frameshift site) in the *core* frame. The SP6 core plasmid DNA was constructed in pSP65; it contains only part of the *pol* gene and has no eukaryotic promoter. The phenotypes of each construct as assayed in tissue culture are shown to the right. **b**, Complementation assay by DNA-DNA and RNA-DNA cotransfection. Cytoplasmic cores were prepared from transfected HepG2 cells by immunoprecipitation. In the left panel, lanes are shown in pairs; for each pair (wt, 1, and 2) the left lane shows DNA prepared from immunoprecipitated cores. The right lane shows the same core preparations extracted with phenol before proteolysis; loss of DNA from the aqueous phase indicates its association with a 5' terminal protein³⁵. Lanes 1, Dfs1 plus DpA1 DNA; lanes 2, Dfs1 DNA alone. In the right panel: lane 1, SP6 core RNA plus Dfs1 DNA; lane 2, SP6 core DNA plus Dfs1 DNA. m, The DHBV 3 kbp linear marker; wt, wild-type.

Methods. For the construction of Dfs1, the unique restriction site *Nsi*I (67 codons into the *core* gene) in the *core* gene of the genomic DHBV clone was filled in by bacteriophage T4 polymerase and recircularized with T4 ligase. For the construction of SP6 core clone, a DNA fragment containing the complete *core* gene from the *Acc*I site (in the pre-C region) to *Ava*I site (downstream of the C gene) was cloned into pSP65 under control of the SP6 promoter.

that bypass the upstream AUGs during scanning from the 5' cap become available to the *pol* AUG. The *pol* initiator, however, is itself in a suboptimal sequence context for initiation, with a C residue rather than a purine in the +4 position (see Fig. 3a). Also, in the 660 nt of the pregenomic RNA 5' to this initiator, there are 15 other AUGs in all three reading frames (not including the *pre-core* AUG). These considerations argue against a simple extension of the leaky scanning model. In addition, experiments with the *core* frameshift mutant Dfs1 also rule out the possibility of ribosomes 'reaching back' for the *pol* AUG after terminating at the *core* terminator. Such 'backwards scanning' has been demonstrated in certain other bicistronic mRNAs with overlapping genes³⁰, but in Dfs1 *core* translation terminates 112 codons upstream of the *pol* AUG, and yet expression of *pol* is unaffected (Fig. 4).

We favour a model of *de novo* initiation at the *pol* AUG. One possibility is that the region flanking the *pol* initiator may have features that facilitate the direct entry of ribosomes or binding of translation initiation factors. Such sequences have been proposed for the cap-independent translation of poliovirus mRNA in which translation initiation occurs at an AUG downstream of an approximately 750-nt region containing seven other AUGs³¹. The use of the *pol* AUG, by whatever mechanism, seems to be low efficiency, as only minute quantities of polymerase protein have been detected in virions by direct biochemical and immunologic tests^{16,17}. This may be of importance in viral gene regulation, as in retroviruses mutations which lead to overexpression of the *pol* precursor are highly deleterious to viral growth³².

A growing number of examples exists of cellular messages which may use internal initiation. A recently described transcript of the human *c-abl* oncogene³³, for example, has a 5' noncoding region of over 1 kb, with 13 AUGs; a similar situation exists upstream of an *Antennapedia* transcript in *Drosophila*³⁴. It is reasonable to assume that these unusual coding organizations have potential regulatory significance. The further study of HBV *pol* and other eukaryotic genes displaying internal initiation should allow a better mechanistic understanding of this intriguing process.

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posons in that the reverse transcriptase function is not generated as a polypeptide with upstream nucleocapsid domains. A similar mechanism of *pol* generation has, however, been suggested for the cauliflower mosaic virus of plants²⁷, in which the *pol* gene can be separated from the upstream cistron without deleterious effects.

In all elements yet encountered that carry out reverse transcription, the reaction is confined to a particulate nucleoprotein structure that presumably serves to prevent the enzyme from gaining access to cellular mRNAs. In retroviruses, frameshifting also solves the problem of selective inclusion of the *pol* protein into viral particles, as the *gag* domain of the *gag-pol* fusion protein can interact with other *gag* proteins during assembly²⁸. In the light of our results, it is clear that hepadnaviruses must solve this problem in some other way, for instance by protein-protein interactions between *core* and *pol*, or by direct interactions between the *pol* protein and pregenomic RNA.

Our data (and those of H. Schlicht, G. Radziwill and H. Schaller, personal communication) indicate that the HBV polymerase is synthesized by initiation at the first *pol* AUG, a view which is further corroborated by the conservation of this AUG in all members of the hepadnavirus family. Such initiations would probably occur from the internal position of the *pol* AUG in pregenomic RNA. Several models for this initiation can be envisioned: the most orthodox interpretation is as an extreme example of 'leaky' scanning²⁹, in which only the rare ribosomes

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Soluble CD4 blocks the infectivity of diverse strains of HIV and SIV for T cells and monocytes but not for brain and muscle cells

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The CD4 antigen has been subverted as a receptor by the human and simian immunodeficiency viruses (HIV-1, HIV-2 and SIV)¹⁻⁴. Several groups⁵⁻⁹ have reported that recombinant, soluble forms of the CD4 molecule (sCD4) block the infection of T lymphocytes by HIV-1, as CD4 binds the HIV envelope glycoprotein, gp120, with high affinity^{10,11}. We now report that sCD4 blocks diverse strains of HIV-1, HIV-2 and SIV, but is less effective for HIV-2. The blocking effect is apparent even after adsorption of virions to CD4 cells. Soluble CD4 prevents HIV infection of T-lymphocytic and myelomonocytic cell lines, but neither sCD4 nor anti-CD4 antibodies inhibit infection of glioma and rhabdomyosarcoma cell lines.

We first investigated the capacity of sCD4 to block infection of diverse strains of HIV-1, HIV-2 and SIV. Table 1 shows that sCD4 preparations blocked the infectivity of each strain tested, but the HIV-2 isolates required 25-fold higher concentrations of sCD4 than HIV-1 to achieve comparable virus neutralization. In a separate experiment, virus strains were treated with sCD4 and titrated for surviving plaque-forming units (p.f.u.) on MT-4 cells¹² with the same results. Table 1 also shows that syncytium formation between HIV-producing and uninfected cells¹ was also inhibited, but was less sensitive to sCD4 than the neutralization of HIV or SIV virions was. This difference may result from the higher viral antigen 'load' present in the cell fusion reaction, which is also less sensitive to most neutralizing antibodies¹³. The relative sensitivity to sCD4 inhibition of the different virus strains was similar for syncytium formation and HIV titration.

The difference in sensitivity to sCD4 between HIV-1 and HIV-2 may reflect a high concentration of free gp120 in HIV-2 stocks, a higher density of gp120 on HIV-2 virions, or a higher binding affinity between HIV-1 gp120 and CD4. We used vesicular stomatitis virus (VSV) pseudotypes bearing the envelope glycoproteins of HIV to investigate whether free gp120

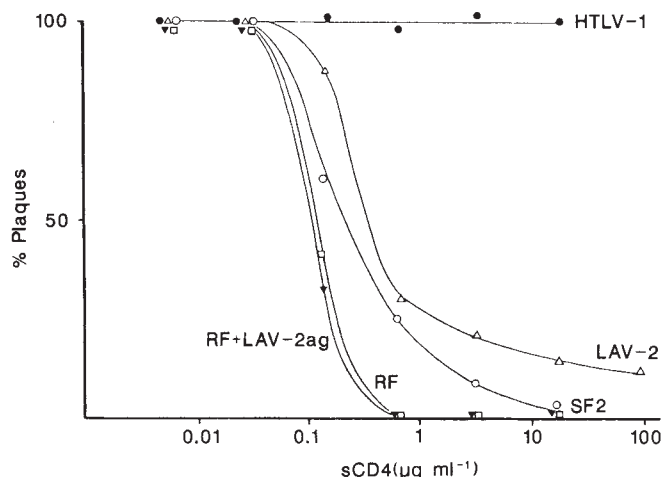


Fig. 1 Inhibition of VSV(HIV) plaque formation by sCD4. VSV pseudotypes bearing the envelope glycoproteins of HIV and HTLV-1 were prepared as described^{1,19,35}. Pseudotype stocks were diluted to 10^3 p.f.u. ml^{-1} and were incubated for 1 h at 37 °C with sCD4 at the concentrations indicated. The reduction in plaque count was scored after assay on adherent CEM cells¹. HTLV-1 was the PL strain³⁵, LAV-2_{ROD} represented HIV-2, HTLV-IIIRF (RF) and SF2 (ARV-2) represented HIV-1 strains. Undiluted LAV-2_{ROD} supernatant medium (LAV-2 ag) was added to VSV(RF) to test the effect of excess HIV-2 antigen on plaque inhibition by sCD4.

competed with sCD4 neutralization of virions. VSV(HIV) pseudotypes depend on CD4 surface receptors for binding to and entry into host cells¹. Once inside the cell, however, the pseudotype replicates as VSV, providing a simple plaque assay for viral antigen-cell receptor interactions.

Figure 1 shows that sCD4 was effective in neutralizing both HIV-1 and HIV-2 pseudotypes. The pseudotype of the HIV-1 strain (HTLV-IIIRF) however was more sensitive to sCD4 than the HIV-2 strain tested (LAV-2), and a second HIV-1 strain (SF2, formerly known as ARV-2) was intermediate. SF-2 represents an isolate that is unusually sensitive to antibody neutralization by human sera¹³, and it may react with sCD4 differently from other HIV-1 strains. The 80% blocking titres of sCD4 for VSV(HIV) pseudotypes were similar to those for HIV infectivity, being approximately $0.3 \mu\text{g ml}^{-1}$ for VSV(HTLV-IIIRF) and $5 \mu\text{g ml}^{-1}$ for VSV(LAV-2). As a control for the specificity of sCD4 neutralization, VSV(HTLV-1) pseudotypes were similarly reacted with sCD4 and were not inhibited at any concentration.

Evidence that the observed difference in sCD4 sensitivity between HIV-1 and HIV-2 is not related to soluble gp120 was obtained from a competition experiment in which supernatant medium from an LAV-2 releasing culture was added to VSV(HIV-1) before sCD4 treatment. The HIV-2 containing medium should have no effect on the VSV(HIV) plaque count, but should compete with VSV(HIV) particles for binding sCD4. LAV-2 supernatants were added to VSV(HTLV-IIIRF) at a concentration that required at least $40 \mu\text{g ml}^{-1}$ sCD4 for its infectivity to be inhibited. Despite this 'overload' of HIV-2 antigen, there was no reduction in sensitivity of VSV(HTLV-IIIRF) to sCD4 inhibition (Fig. 1). Addition of HIV-1 supernatant had no effect on VSV(HIV) inhibition either (data not shown).

We next investigated whether sCD4 would inhibit HIV after virions had bound to cellular CD4 receptors. Serial dilutions of HIV-1 were treated with sCD4 either before adsorption or one hour after adsorption to T cells. The results (Table 2) demonstrate the potential of sCD4 to block HIV post-adsorption, albeit some 50-fold less efficiently than if added to HIV before adsorption. The blocking effect indicates that even after binding to

Table 1 Soluble CD4 inhibition of infection and syncytium formation by HIV-1, HIV-2 and SIV strains

		Inhibition titre of sCD4 ($\mu\text{g ml}^{-1}$)		
Virus		Infection TCID	Plaque assay	Syncytium formation
HIV-1	HTLV-IIIB	0.3	0.3	1.5
	HTLV-IIIRF	0.3	0.3	1.5
	SF33	0.12	0.12	0.6
	CBL4	0.3	ND	1.5
HIV-2	LAV-2 _{ROD}	7.6	7.6	38
	CBL20	7.6	7.6	38
SIV	SMM (mangabey)	0.6	ND	7.6
	MAC (macaque)	0.6	ND	7.6
	AGM (green monkey)	0.6	ND	7.6

Soluble CD4 representing the whole of the extracellular domain was prepared as previously described⁸. For infection inhibition (neutralization) assays, serial dilutions of sCD4 were incubated at 37 °C for one hour with 1,000 T-cell infectious doses (TCID) of HIV or SIV. The surviving infectious virus was titrated by plating dilutions of the sCD4-treated virus on C8166 T cells and recording virus replication by the appearance of syncytia and reverse transcriptase activity 48–72 hours later¹⁹. Virus treated with sCD4 was also titrated in the MT4 cell HIV plaque assay¹². For syncytium formation, chronically infected H9 cells were mixed with uninfected C8166 indicator cells and multinucleated syncytia were counted after incubation overnight¹⁹. The concentration of sCD4 neutralizing over 80% of TCID and p.f.u., and causing 80% inhibition of syncytium formation are tabulated for each virus strain. ND, not determined.

cellular CD4, HIV infection can be inhibited by the competing soluble homologue, before internalization by a pH-independent fusion process^{14–16}.

HIV infection of T-lymphocytic^{1,2,17} and monocytic^{18,19} cells can be blocked by preincubating the cells with certain anti-CD4 monoclonal and polyclonal antibodies. Other cell types, notably glioma cell lines^{20–22} and RD cells²³, can also be infected with high titres of HIV-1. HIV-susceptible glial cell lines, such as U138, are negative for CD4 surface expression by immunofluorescence, but express low levels of CD4 mRNA. (J.N.W., unpublished observations). Preliminary experiments indicated that anti-CD4 antibodies did not inhibit HIV infection. The low density of CD4 expression on glial cells might prevent anti-CD4 attaching with sufficient avidity to compete with HIV binding. By using sCD4 as an inhibitory reagent binding to the virion glycoproteins, gp120, rather than to the cellular receptor molecule, the question of whether CD4 is required for infection of these cells can be addressed more clearly.

Table 3 shows that U138 astrogloma cells, but not U87 astrogloma cells, can be infected with the HTLV-IIIRF strain of HIV-1. The TE671 cell line can also be infected with HIV²⁰. This line was thought to be derived from a human medulloblastoma²⁴, but is now known to be the RD rhabdomyosarcoma cell line²⁵. A relatively high input of virus required for U138 and RD cell infection ($>10^3$ T-lymphocyte infectious units), and the infection is only weakly productive. Not enough virions are released into the supernatant medium of infected U138 or RD cultures to register as positive in reverse transcriptase or p24 antigen assays, and it seems that only a small proportion of cells contain HIV antigens or proviral DNA^{20–22}. Virus can readily be rescued by cocultivation with a fully permissive T-cell line however, such as C8166 or by transfer of the supernatant medium of infected glioma cell cultures to C8166 cells. Incubation of HIV-1 with sCD4 before plating failed to block infection of brain or muscle cell lines at $18 \mu\text{g ml}^{-1}$ or even $180 \mu\text{g ml}^{-1}$. In a parallel experiment, monoclonal antibodies reacting to CD4 antigen (Leu3a, F101-5) efficiently blocked HIV infection of CD4⁺ lymphocytic and monocytic cells but also failed to block

Table 2 Reduction of HIV-1 infection by sCD4 after adsorption of virus to T-lymphocytes scored by syncytial focus assay

Virus dilution	No. sCD4	sCD4 added before HIV adsorption	sCD4 added after HIV adsorption
10^0	con, con	1, 2	105, 85
10^{-1}	203, 240	0, 0	10, 13
10^{-2}	25, 41	0, 0	3, 1
10^{-3}	4, 2	0, 0	0, 0

HIV-1 (HTLV-IIIRF) was titrated by a syncytial focus assay as follows: C8166 cells (10^6 cells in 0.5 ml RPMI/1640 medium with 10% fetal calf serum) were plated into 12-mm tissue culture wells in 24-well plate wells (Nunc, Denmark) coated with $50 \mu\text{g ml}^{-1}$ poly-L-lysine to form a monolayer. After 20 min at room temperature, appropriate virus dilutions were added and adsorbed for 1 h on ice. The monolayers were washed in culture medium to remove unadsorbed virus and were incubated in medium for 1 h, washed again, and overlaid with 1 ml culture medium containing 0.9% molten Sea Plaque agarose (FMC Bioproducts). After 42 h incubation at 37 °C, 1 ml agarose containing 0.0032% Neutral Red was added and at 48 h discrete focal areas of syncytia were counted by low power magnification ($\times 20$). The linear dose-response of syncytial foci in the absence of sCD4 indicated that each focus resulted from a single infectious unit of HIV and the focus titre correlated well with HIV titration by end-point dilution on C8166 cells in suspension (TCID₅₀). To measure the inhibitory effect of sCD4, $40 \mu\text{g ml}^{-1}$ protein was added to serially diluted HIV for 1 h on ice in culture medium before adsorption, or was added to the washed cell monolayer on ice following virus adsorption. The numbers represent focus counts of duplicate wells for each sCD4 treatment and virus dilution. Con, confluent syncytia.

infection of brain tumour cells. In contrast, a human serum containing antibodies to gp120, gp41 and gag antigens neutralized HIV for all cell types (Table 3). It is most unlikely that input virus has been carried over in the brain cell cultures, in view of the serial passage and trypsinization of these cells, and that the resistant U87 glial cell line did not yield rescued virus upon subsequent cocultivation with T cells (Table 3). Moreover, proviral DNA has been detected after HIV infection of these cell types^{20,21}. We therefore conclude that HIV infects glial and muscle cells in a way that is not mediated by CD4.

Soluble CD4 is under investigation as a therapeutic agent in HIV-infected subjects. One concern is that sCD4 might itself be immunosuppressive, reacting *in vivo* with the apparent natural ligand for CD4, the major histocompatibility complex (MHC) class II antigens^{4,26}. Little or no effect of sCD4 on class II-restricted T-cell interactions is seen *in vitro* however⁷. The primary recognition site on CD4 for gp120 is close to the Leu3a antigenic epitope^{4,27} and has been mapped to the N-terminal immunoglobulin V-like domain^{28,29}. An sCD4 fragment or peptide containing this site but which was not involved in immune recognition would be desirable, though the evolutionary conservation among primate species of the gp120/Leu3a site suggests that this epitope may be important for normal functions of the CD4 molecule³⁰.

Our results show that neither sCD4 nor Leu3a antibody block infection of certain brain and muscle cell lines. These unexpected results indicate that HIV may not require cell surface CD4, nor perhaps viral gp120, to gain entry to these cells *in vitro*. It would seem unlikely to depend on an artefactual 'transfection' as described for bacteriophage³¹ as DEAE-dextran was not used to enhance virion uptake, and the RD/TE671 cultures became infected with an input as low as 0.05 T-cell infectious doses per cell (Table 3). HIV and VSV(HIV) pseudotypes are unable to infect mouse cells expressing human CD4 or to induce cell fusion although the virions bind to the CD4 molecules³². A second membrane molecule may therefore be required for fusion and viral penetration. In view of the relatively low efficiency of infection of glioma and myoblast cells compared to CD4⁺ T

Table 3 Effect of sCD4 and anti-CD4 antibody on HIV-1 infection of different human cell types

Cell type	Input HIV (TCID)	Human anti-HIV-1		Leu3a anti-CD4		sCD4 180 µg ml ⁻¹		Control	
		SF	RT	SF	RT	SF	RT	SF	RT
T cell									
C8166	5 × 10 ³	—	—	—	—	—	—	+++	20,000
Myelomonocyte									
U937	5 × 10 ⁵	ND	ND	—	—	—	—	+++	27,000
	5 × 10 ³	—	—	—	—	—	—	+++	55,000
HL60	5 × 10 ⁵	ND	ND	—	—	—	—	++	49,000
	5 × 10 ³	—	—	—	—	—	—	++	9,000
Glioma									
U138	5 × 10 ⁵	—	—	++	21,000	++	23,000	++	23,000
U87	5 × 10 ⁵	—	—	—	—	—	—	—	—
Muscle									
RD/TE671	5 × 10 ⁵	—	—	++	18,000	++	26,000	++	19,000
	5 × 10 ³	—	—	++	9,000	++	20,000	++	18,000

HIV-1 (HTLV-IIIRF strain) was incubated with anti-HIV-1 antibody or with sCD4 for 1 h at 37 °C before plating on 10⁶ cells of the types indicated. Syncytium formation (SF) and reverse transcriptase (RT) activity in the medium was scored 5 days post-infection for the T-cell and myelomonocyte cultures. For the glial and myoblast cultures there were no detectable SF or RT activity as previously described²⁰⁻²³. After seven days and two trypsin-mediated passages of the glial and myoblast cells, C8166 cells were added to the cultures, and SF and RT activity was scored 3 days later. Virus production for cells exposed to HIV-1 (not treated with anti-CD4 or sCD4) was also detected after 3 weeks and 6 passages in U138 and RD/TE671 cells, but not U87 cells. The glial cells expressed glial fibrillar acidic protein, confirming their provenance. SF scores: +++, >50% giant syncytial cells in culture; ++, 10-50% syncytial cells; +, 1-9% syncytial cells; —, no discernable syncytia. RT scores in c.p.m. acid precipitable [³H]thymidine incorporation; —, <1,000 c.p.m. ND, not determined.

lymphocytes, HIV infection might occur by gp41-mediated membrane fusion in the absence of specific gp120-CD4 receptor interaction.

It is not clear whether HIV infection of astroglial cells has a role in HIV encephalopathy (as infection is mainly detectable in microglial and endothelial cells^{33,34}) or whether muscle infection is related to the weight loss and wasting seen in AIDS. Although our results may be restricted to cells *in vitro*, HIV infection of glial and muscle in the presence of excess sCD4 merits further investigation, given the interest in developing sCD4 as a therapeutic agent.

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Productive dual infection of human CD4⁺ T lymphocytes by HIV-1 and HHV-6

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Although infection by HIV-1 (refs 1 and 2) has been implicated as the primary cause of AIDS and related disorders^{3,4}, cofactorial mechanisms may be involved in the pathogenesis of the disease. For example, several viruses commonly detected in AIDS patients and capable of transactivating the long terminal repeat of HIV-1, such as herpesviruses⁵⁻⁷, papovaviruses⁷, adenoviruses⁸ and HTLV-I (ref. 9), have been suggested as potential cofactors. Another candidate is human herpesvirus-6 (HHV-6, originally designated human B-lymphotropic virus)¹⁰⁻¹², which has not only been identified in most patients with AIDS by virus isolation^{10,13,14}, DNA amplification techniques¹⁵ and serological analysis¹⁶, but is also predominantly tropic and cytopathic *in vitro* for CD4⁺ T lymphocytes^{17,18}. Here we demonstrate that HHV-6 and HIV-1 can productively co-infect individual human CD4⁺ T lymphocytes, resulting in accelerated HIV-1 expression and cellular death. We also present evidence that HHV-6 transactivates the HIV-1 long terminal repeat (LTR). These observations indicate that HHV-6 might contribute directly or indirectly to the depletion of CD4⁺ T cells in AIDS.

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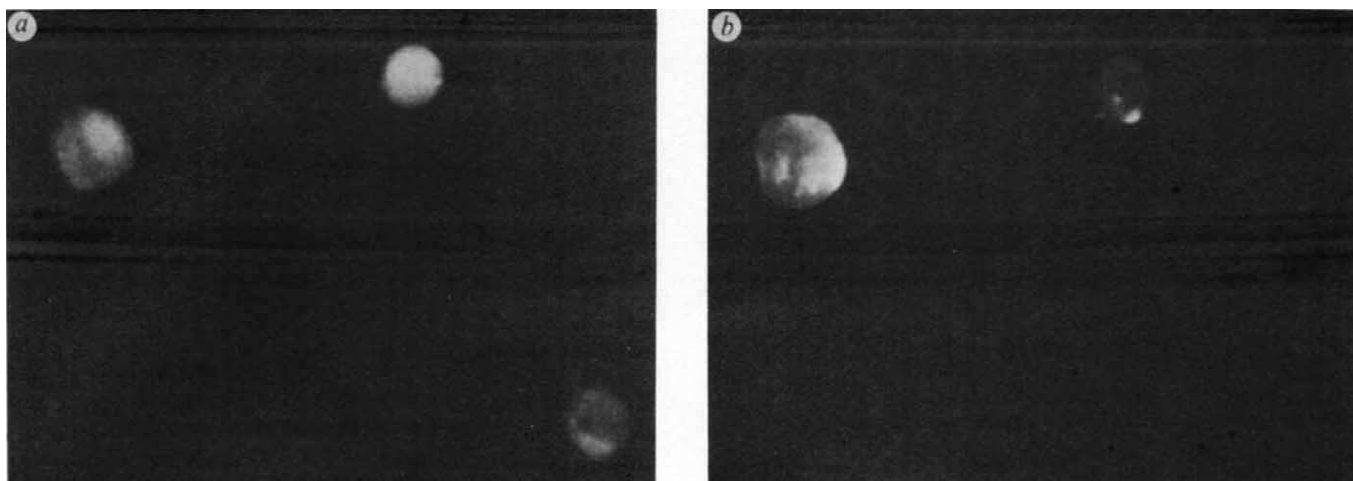
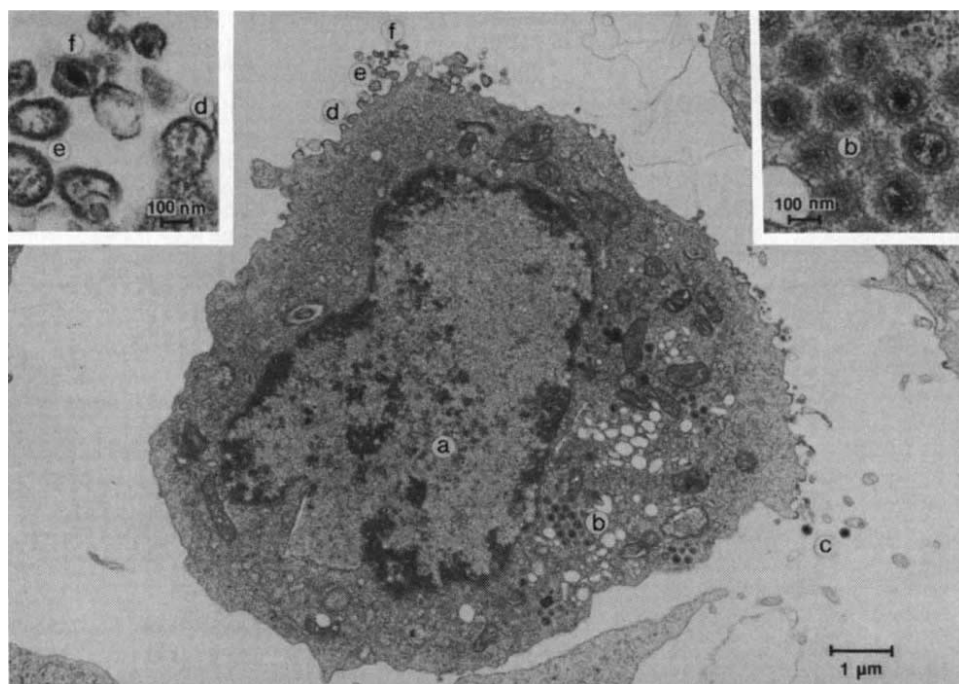


Fig. 1 Simultaneous expression of specific antigens of HHV-6 (*a*) and HIV-1 (*b*) in doubly infected cell cultures, as assessed by two-colour indirect immunofluorescence.

Methods. PBMC from healthy volunteers were isolated by Ficoll-Hypaque gradient centrifugation and cultured at 37 °C in a humidified atmosphere of 5% CO₂ at a density of $1 \times 10^6 \text{ ml}^{-1}$ in RPMI medium supplemented with 15% fetal bovine serum, $1 \mu\text{g ml}^{-1}$ phytohaemagglutinin-P and $5 \mu\text{g ml}^{-1}$ polybrene. Cells were washed with RPMI after 24 h, counted and exposed for one hour at 37 °C to HIV-1 alone (isolate HTLV-III_B), HHV-6 alone or to HIV-1 plus HHV-6; each virus was at an infectious multiplicity of ~ 0.3 . Infected cells were then cultured at 10^6 per ml in RPMI supplemented with 10% fetal bovine serum and 20% of their original culture medium collected before infection. The absolute numbers of viable cells, CD4⁺ cells and giant multinucleated cells (syncytia) were recorded at 2-day intervals. After removal of dead elements by Ficoll-Hypaque gradient centrifugation, viral antigen expression was evaluated in parallel by indirect immunofluorescence on cytocentrifuge preparations fixed for 5 min in cold acetone¹⁸. A murine monoclonal antibody against the gag p24 protein of HIV-1 (M26)²⁰ was used at $2 \mu\text{g ml}^{-1}$; an anti-HHV-6 human serum (N828), extensively adsorbed with uninfected phytohaemagglutinin-activated PBMC, was used at a final dilution of 1:15. Phycoerythrin-conjugated (red) goat anti-mouse IgG and fluorescein-isothiocyanate-conjugated (green) goat-anti-human IgG polyclonal antisera (both specific for γ -chain; Kirkegaard and Perry Inc., Maryland), were used as second layer antibodies. Samples were visualized with a Leitz Ortholux II fluorescence microscope equipped with a $\times 40$ dry objective. Two-colour immunofluorescence also demonstrated that cells infected with HHV-6 and HIV-1 displayed a CD4⁺ T-cell phenotype.

Fig. 2 Electron micrograph of a cell with the morphological features of an activated lymphocyte, eight days after simultaneous infection of PBMC cultures with HIV-1 and HHV-6. *a*, Unenveloped intranuclear HHV-6; *b*, unenveloped intracytoplasmic HHV-6; *c*, mature extracellular HHV-6; *d*, budding HIV-1 particles; *e*, immature extracellular HIV-1 (rarefied core); *f*, mature HIV-1 (dense core). Magnification is $\times 15,000$ (detailed inserts, $\times 75,000$). Cells were extensively washed with cold PBS, centrifuged at 1,500 r.p.m. and fixed with 1.25% glutaraldehyde, 0.1 M sodium cacodylate, $0.5 \times \text{PBS}$ for electron microscopy.



Freshly isolated normal human peripheral blood mononuclear cells (PBMC) were activated *in vitro* with purified phytohaemagglutinin and subsequently co-infected with HIV-1 and HHV-6. Two-colour indirect immunofluorescence was used to detect specific viral antigens and demonstrated that individual cells could serve as a simultaneous target for both viruses (Fig. 1). Electron microscopy illustrated the productive nature of this dual infection, showing the complete pathway of HHV-6 and HIV-1 maturation within individual lymphocytes. HHV-6 was visible as both immature (namely, nuclear or cytoplasmic unen-

veloped nucleocapsids) and mature extracellular particles (Fig. 2*a-c*). Budding, extracellular immature and mature HIV-1 virions could be recognized if associated with the same cell (Fig. 2*d-f*).

The expression of viral messenger RNA, as analysed by northern blotting, was qualitatively unaltered in dually infected cultures and the viruses released were found to be infectious for normal human PBMC (data not shown). As summarized in Table 1, however, HIV-1 antigen expression was consistently accelerated in the course of co-infection with HHV-6. It must

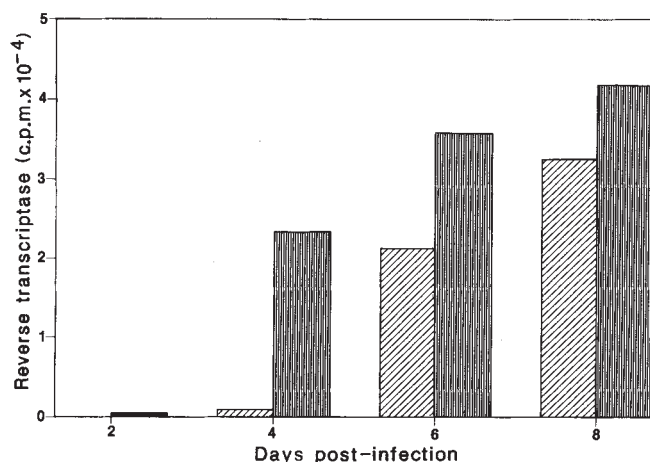


Fig. 3 Reverse transcriptase activity in cultures of PBMC infected with HIV-1 alone (cross-hatched) or with HIV-1 and HHV-6 together (shaded). Details of infection and culture procedures are given in the legend to Fig. 1. Polyethyleneglycol-precipitated cell culture supernatant fluids were assayed for reverse transcriptase activity as previously described².

be emphasized that the majority of HIV-1-positive cells were simultaneously expressing HHV-6 antigens. Moreover, these immunofluorescence determinations probably underestimated the actual magnitude of the infection, as dead (presumably infected) cells were removed before testing. An accelerated expression of HIV-1 in dually infected cultures was further indicated by the earlier detection of reverse transcriptase activity (Fig. 3).

Consistent with the acceleration of HIV-1 expression, we also observed a more rapid induction of cytopathicity in the course of dual infection. The loss of CD4⁺ T lymphocytes was significantly accelerated in dually infected PBMC cultures (Fig. 4a) and mathematical analysis of the data suggested a synergistic cytotoxic action of HIV-1 and HHV-6 (Fig. 4b). A parallel decline of total cell viability was also observed in these cultures. For example, eight days after infection, the absolute numbers of viable cells were $4.3 \pm 0.5 \times 10^5$ with HIV-1 and $2.7 \pm 0.5 \times 10^5$ with HHV-6, as compared to $0.6 \pm 0.2 \times 10^5$ with both viruses together ($P < 0.01$). These observations indicate that, despite the documented down-regulation of CD4 molecules by HIV-1 gp120/160 glycoproteins¹⁹, the chief mechanism for CD4⁺ cell depletion in our cultures was a virus-induced cytopathic effect. Moreover, HIV-1 induced syncytia were identified earlier in doubly infected cultures (Table 1). Dual infection of CD4⁺ T cells was also observed using established human CD4⁺ T cell lines (not shown).

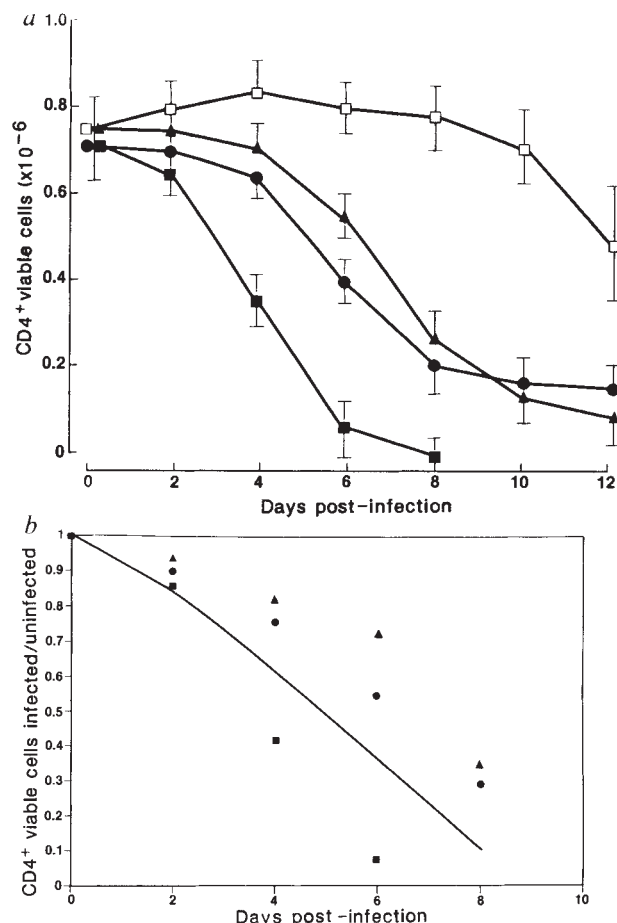


Fig. 4 Cytopathic effect in the course of co-infection of normal human PBMC by HIV-1 and HHV-6. Open square, uninfected; filled triangle, HIV-1 alone; filled circle, HHV-6 alone; filled square, HIV-1 plus HHV-6. *a*, Kinetics of CD4⁺ cell viability in singly and doubly infected cultures. Data represent the averaged results ($\pm$ standard deviation) from 3 separate experiments. *b*, Synergistic effect of HIV-1 and HHV-6 in inducing cytopathicity on normal PBMC cultures. All values are normalized with respect to uninfected control cultures (representing the ratio between viable CD4⁺ cell counts in infected cultures and viable CD4⁺ cell counts in uninfected cultures for each time point). The solid line represents the predicted curve in the course of an additive effect and was calculated according to the following formula: $N_t = p_t q_t$ (where p_t and q_t correspond to normalized CD4⁺ cell counts at time t in cultures infected with HIV-1 and HHV-6 respectively). By day 4 or 6 post-infection, the observed mortality in dually infected cell cultures was 2–3 times greater than the values predicted for a simple additive effect.

Table 1 Viral antigen expression and syncytia formation in normal PBMC cultures infected with HIV-1, HHV-6 or both viruses simultaneously

Day post-infection	Virus used for infection*							
	HIV-1		HHV-6		HIV-1 plus HHV-6		S‡	
	HIV-1† %	S‡	HHV-6† %	S‡	HIV-1† %	HHV-6† %		
2	—	—	—	—	0.7 (0.6)	—	—	—
4	1.7 (1.6)	—	6.7 (5.8)	—	10.7 (7.5)	14.7 (10.3)	9.3 (6.5)	—
6	9.3 (7.2)	—	30.7 (14.4)	—	38.3 (12.6)	45.7 (15.1)	24.0 (7.9)	+
8	22.7 (9.7)	+	68.3 (18.4)	—	61.0 (3.7)	82.7 (5.0)	50.3 (3.0)	+
10	30.3 (11.2)	+	80.0 (18.4)	—	NA	NA	NA	+

* Phytohaemagglutinin-activated PBMC were infected with HIV-1, HHV-6 or both viruses simultaneously. Details of the infection procedure and indirect immunofluorescence assay are given in the legend to Fig. 1.

† Per cent immunofluorescent-positive viable cells and in parenthesis absolute numbers of positive viable cells $\times 10^{-4}$ (averaged results from three separate experiments) for the indicated virus, determined as described in the legend to Fig. 1.

‡ Presence (+) or absence (–) of syncytia (S) as evaluated on Wright–Giemsa stained cytocentrifuge preparations, each containing 2×10^5 cells.

|| NA, not applicable. In these cultures no viable cells were detected by day 10 post-infection.

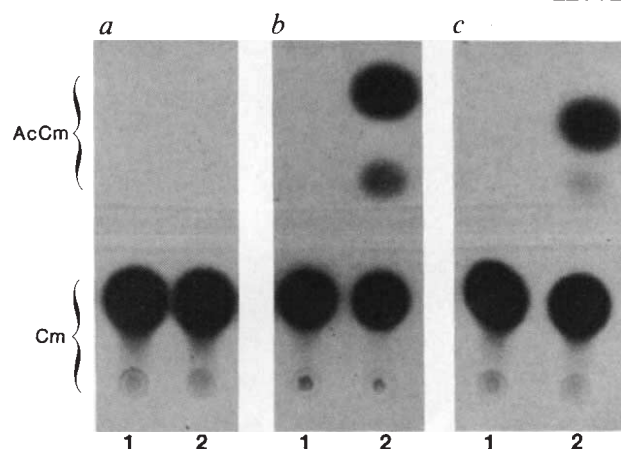


Fig. 5 Transactivation of the chloramphenicol acetyltransferase (CAT) gene linked to the HIV-1 LTR when transfected into MOLT-3 cells which were uninfected (a), infected with HIV-1 (b) or infected with HHV-6 (c). Plasmids were pC15CAT (lanes 1; ref. 21) containing the HIV-1 LTR linked to the bacterial CAT reporter gene, and the control pSVOCAT (lanes 2; ref. 22) that lacked promoter-enhancer sequences.

Methods. 10^7 cells were transfected with 10 μ g plasmid DNA by the DEAE-dextran technique²³, collected after 40 h and processed to obtain 100 μ l cellular extract; aliquots of 20 μ l were subsequently used for CAT assays in a 3-h incubation²⁴. Different percentages of cell viability were found in the various cultures at the time of collection: a1, 79%; a2, 89%; b1, 51%; b2, 49%; c1, 26% and c2, 29%. The acetylation of chloramphenicol (%) as estimated from liquid scintillation counting was a1, 0.19; a2, 0.24; b1, 2.5; b2, 30; c1, 0.27 and c2, 19.7. Cm, chloramphenicol; AcCm, acetylated chloramphenicol.

The accelerated virus expression and cytopathicity in dually infected cultures suggested that a direct interaction occurs between HIV-1 and HHV-6. We therefore studied the transactivation of the HIV-1 LTR by HHV-6 using the human CD4⁺ T-cell line MOLT-3. As illustrated in Fig. 5, there was a consistent transactivation in HHV-6-infected cells which was not found in uninfected cells. We obtained similar results using the CD4⁺ T-cell line CEM (data not shown).

The part played by cofactorial mechanisms in the pathogenesis of AIDS and in particular the relevance of other potentially immunosuppressive agents are still to be clarified. We have previously demonstrated that HHV-6 exerts a direct lytic effect on human CD4⁺ T lymphocytes¹⁸. Here we have shown that HHV-6 can co-infect individual T cells with HIV-1 and can transactivate the expression of HIV-1. The simultaneous presence of both viruses in the same cell emphasizes the potential importance of this transactivation. Replication of HHV-6 might in turn be enhanced by co-infection with HIV-1, thus generating a vicious cycle. Our observations, although they do not prove the aetiological involvement of HHV-6, are consistent with a possible cofactorial role of this virus in one of the critical pathological features of AIDS, namely the CD4⁺ T-cell depletion. Prospective long-term seroepidemiological and *in vivo* investigations, using animal models or two-probe hybridization to detect dual infection in individual patient cells, will help evaluate the contribution of potential cofactors to the course of HIV-1 infection.

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Genetically haploid spermatids are phenotypically diploid

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Because chromosomal homologues segregate from one another during meiosis, spermatids are genetically different. Post-meiotic gene expression could lead to gametic differences, some of which might lead to preferential transmission of certain alleles over others. In both insects and mammals, however, all the cells derived from a single spermatogonial cell develop within a common syncytium formed as a result of incomplete cytokinesis at each of the mitotic and meiotic cell divisions¹⁻³. It has been proposed that the intercellular bridges connecting the cells, which are about 1 μ m in diameter⁴, permit the sharing of cytoplasmic constituents, thus ensuring the synchronous development of a clone of cells and gametic equivalence between haploid spermatids^{2,5,6}. By analysing the product of a transgene which is expressed exclusively in post-meiotic germ cells in hemizygous transgenic mice, we have shown that genetically distinct spermatids share the product of the transgene and hence can be phenotypically equivalent.

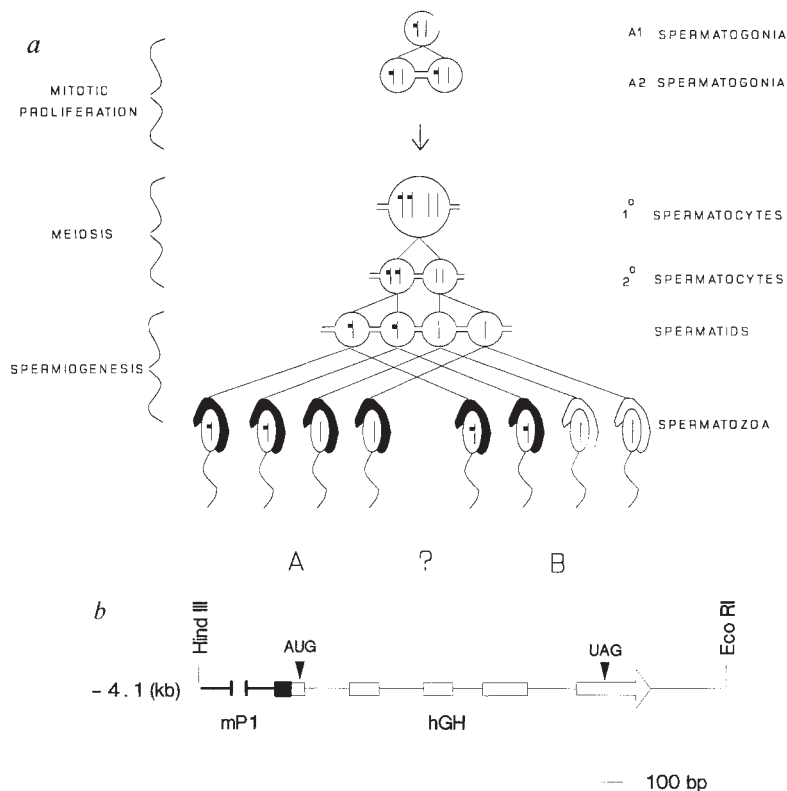
The experiments described here were designed to determine whether either RNA or protein can pass through the intercellular bridges which connect spermatids. To address these questions we have generated transgenic mice that carry a chimaeric gene consisting of the mouse protamine 1 (*mP1*) transcriptional regulatory sequences, fused to the human growth hormone (*hGH*) structural gene. The advantage of using transgenic mice for such an analysis is that mice can be generated which are hemizygous for the transgene, that is, the introduced DNA is present on only one of the two chromosomal homologues, so that after meiosis only half of the spermatids carry the transgene (Fig. 1a). The *mP1* gene is transcribed exclusively during spermiogenesis, the haploid phase of spermatogenesis⁷. Previous studies have shown that 4.8 kilobase (kb) of 5' sequence is sufficient for the proper tissue-specific and temporal regulation of *mP1* in transgenic mice⁸. The construct used in this study contains 4.1 kb of *mP1* 5' sequence fused to the *hGH* gene (Fig. 1b).

Two independent lines of transgenic mice harbouring the *mP1-hGH* gene were established (Fig. 1b). To determine the transmission frequency of the transgene, tail DNA from

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Fig. 1 a, Illustration of the syncytial nature of spermatogenesis and the three general stages of development^{20,21}. The spermatogonial cells are drawn with a pair of homologous chromosomes inside them with one chromosome containing the introduced transgene (shown by a solid box). After the commitment of A1 spermatogonial cells to differentiate there are five or six rounds of mitotic proliferation. The first cell division generating A2 spermatogonial cells is shown. Primary spermatocytes contain a 4N complement of DNA, that is, each chromosome is associated with a sister chromatid. Each primary spermatocyte gives rise to four spermatids, two that contain a wild-type chromosome and two that carry a chromosome with the transgene. During spermiogenesis, round spermatids are transformed into mature spermatozoa. The question is whether all of the spermatozoa contain the product of the transgene (in this case the hGH protein localized to the acrosome), as shown in A, or whether only those spermatozoa that contain the transgene will have the product, as shown in B. **b**, Plasmid *mP1-hGH* contains approximately 4.1 kb of *mP1* 5' sequence, including the promoter and 91 base pairs (bp) of 5' untranslated sequence fused to a genomic clone for the *hGH* gene. Boxes represent exons and lines represent either non-transcribed DNA or introns. Bold lines and the solid box represent *mP1* DNA whereas thin lines and open boxes represent *hGH* DNA. The break in the line that corresponds to *mP1* sequence denotes that portion of the transgene that is not drawn to scale. The positions of the translational start and stop codons in the first and last exon, respectively, are shown above the gene structure. Transgenic mice were constructed by microinjecting a 6.3 kb *HindIII-EcoRI* fragment containing the *mP1-hGH* gene, free of plasmid vector sequences, into the pronuclei of fertilized C57BL/6 $\times$ SJL F2 mouse eggs²². Two lines of transgenic mice, 314-4 and 315-6, were generated and used for this study. Both lines contain multiple copies of the transgene integrated on one chromosome.



offspring was analysed for the presence of the transgene by hybridization with an *hGH*-specific radiolabelled DNA probe. Analysis of the progeny from three generations of outcrosses of transgenic with nontransgenic animals showed that the mice were hemizygous for the transgene (data not shown).

In situ hybridization was used to determine the developmental stage in which the transgene is first transcribed. Figure 2a and b shows serial sections of a testis prepared from a hemizygous transgenic adult male, and hybridized with ³⁵S-labelled antisense RNA probes specific for the *mP1-hGH* transgene and the endogenous *mP1* gene, respectively. In both cases expression was detected exclusively in the haploid spermatids and not in spermatogonial cells or in spermatocytes.

Northern blot analysis of RNA isolated from the testis of prepubertal transgenic animals of different ages supported the conclusion that the transgene was expressed exclusively in post-meiotic cells. No transcripts were detected from the transgene or the endogenous gene in testis from 19-day-old animals, an age at which cells in the first cycle of spermatogenesis have progressed to the pachytene spermatocyte stage^{9,10}, whereas transcripts from both genes were detected, in about equal abundance, in 25-day-old animals, an age at which some cells have progressed into the haploid stage of development (data not shown).

To determine the phenotype of spermatids with respect to the product of the *mP1-hGH* transgene we used immunocytochemical analysis. Spermatozoa from transgenic mice harbouring the *mP1-hGH* construct contain the hGH protein localized in the acrosomal compartment (R.E.B. *et al.*, in preparation). Visualization of the hGH protein in developing acrosomes in early-stage seminiferous tubules (Fig. 3a), and in later-stage tubules (Fig. 3b), showed that the majority of the spermatids within a tubule of a hemizygous mouse were immunopositive for the hGH protein. Immunocytochemistry performed on the caput epididymis (the proximal portion of the epididymis where newly formed spermatozoa reside), showed that the hGH protein

was stable in the acrosome and that, once again, most of the spermatozoa were immunopositive (Fig. 3c).

To quantitate the percentage of hGH-immunopositive spermatozoa, we isolated sperm from the caput epididymis (Fig. 3d) and determined directly the percentage that were immunopositive. Approximately 90% of all caput sperm analysed from hemizygous animals contained hGH-immunopositive acrosomes (Table 1), despite the fact that the gene was transmitted to only 50% of the offspring, showing that post-meiotic gene products can be shared.

Failure to detect hGH protein in 9% of the spermatozoa could be due to incomplete movement of RNA or protein between spermatids, nondetectable levels of expression of the transgene in some spermatids, or technical problems such as loss of the acrosome during the sperm isolation. To distinguish between incomplete movement of RNA or protein between the intercellular bridges and the other possibilities, we analysed caput sperm from mice homozygous for the transgene. Even in homozygous mice only 92% of the sperm were immunopositive (Table 1),

Table 1 Quantitation of the percentage of hGH-immunopositive spermatozoa

Genotype	Total sperm analysed	Percentage immunopositive
Hemizygous	2,185	91%
Homozygous	2,768	92%

Spermatozoa were isolated from the caput epididymis and immunocytochemistry was performed as described in the fig. 3 legend. These results were obtained by analysing spermatozoa isolated from three hemizygous mice (two from the 315-6 line and one from the 314-4 line) and two homozygous mice (both from the 314-4 line). Homozygous mice were obtained by crossing hemizygous transgenic animals and screening the offspring by quantitative DNA dot hybridization with an *hGH*-specific probe. Cells were counted using differential interference contrast microscopy at $\times 400$ magnification.

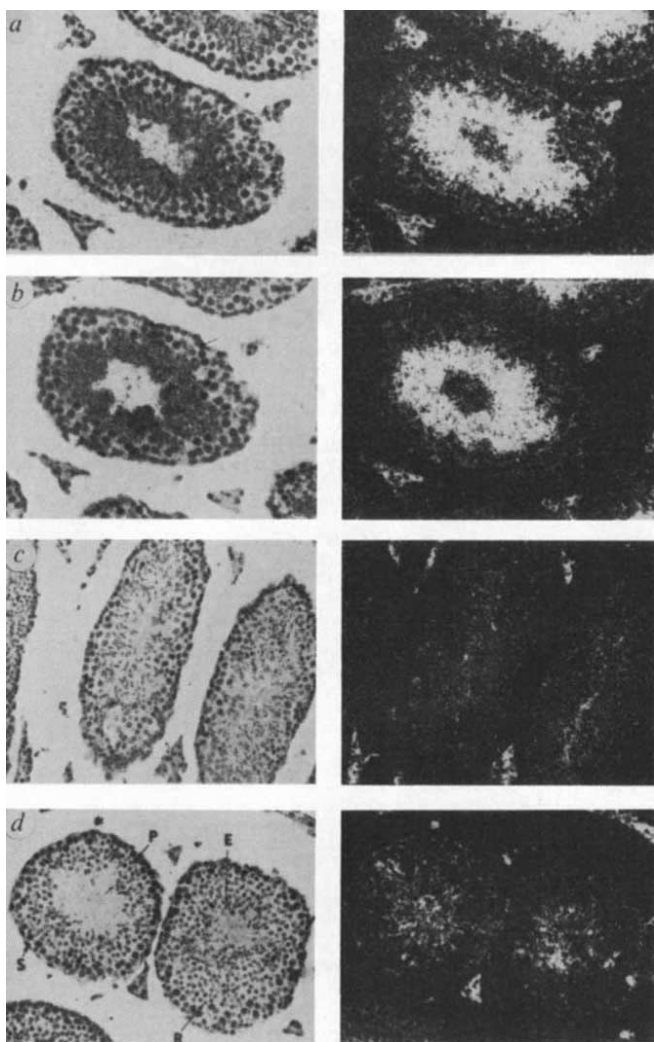


Fig. 2 *In situ* hybridization of testis sections from a transgenic mouse harbouring the *mPI-hGH* gene (line 314-4). Serial sections were hybridized with ^{35}S -labelled probes: *a*, *hGH* antisense RNA; *b*, *mPI* antisense RNA; *c*, *hGH* sense RNA; and *d*, *mPI* sense RNA. Photographs on the left were taken with bright-field microscopy and those on the right with dark-field microscopy. Examples of the different cell types found within a seminiferous tubule are shown by arrows and are labelled as follows: S, spermatogonial cell; P, pachytene spermatocyte; R, round spermatid; and E, elongated spermatid.

Methods. Single-stranded ^{35}S -containing sense and antisense riboprobes were made *in vitro* with linear templates and either SP6 or T7 RNA polymerase. The *mPI* template was made by cloning a 250-bp restriction fragment containing a portion of *mPI* exon 1, the intron, and a portion of exon 2 into pGEM-2. The *hGH* template was constructed by cloning a 170 bp restriction fragment containing *hGH* exon 5 into pGEM-2. Testes were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm sections. Hybridizations were as described²³. Probes were used at a concentration of about $0.2 \text{ ng } \mu\text{l}^{-1}$ with a specific activity of approximately $5 \times 10^5 \text{ c.p.m. ng}^{-1}$. Hybridizations were at 45° overnight. The most stringent washing conditions were at 45° in $0.1 \times \text{SSC}$. Slides were developed after one to three days. Sections were counterstained with haematoxylin.

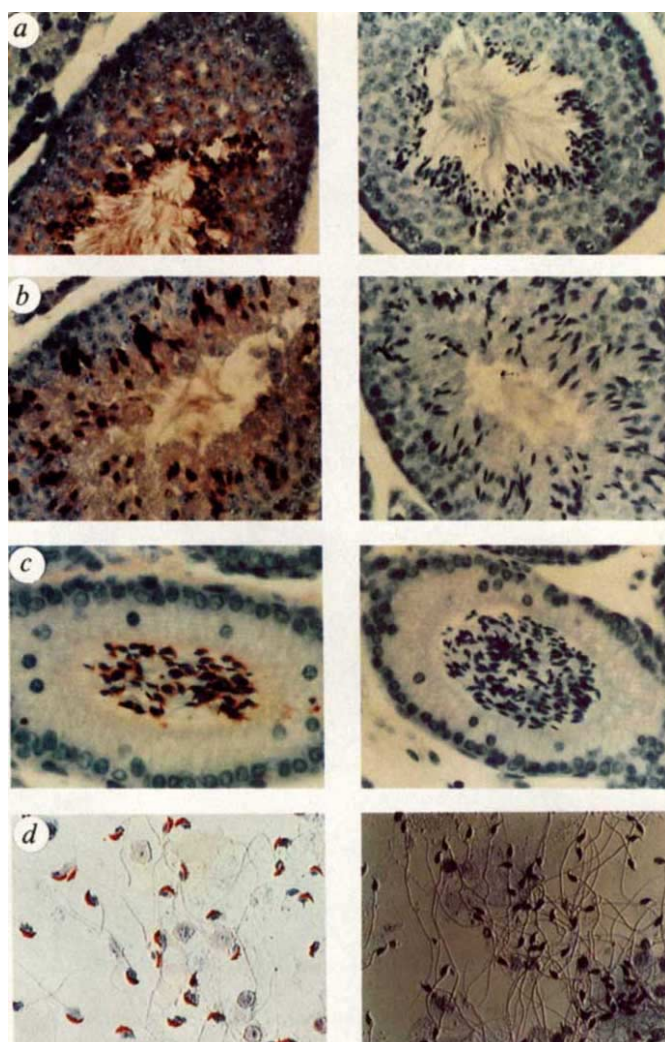


Fig. 3 Immunocytochemistry of *hGH* protein in *mPI-hGH* transgenic mice (line 314-4). Photographs on the left show sections incubated in the presence of both the primary antibody (rabbit anti-*hGH*), and secondary antibody (biotinylated goat anti-rabbit IgG). Photographs on the right show sections incubated only in the presence of the secondary antibody. *a*, An early-stage seminiferous tubule with round spermatids at the CAP phase of acrosome development⁹. *b*, A late-stage tubule with many elongated spermatids and fully developed acrosomes. *c*, A section through the caput epididymis. *d*, Spermatozoa isolated from the caput epididymis. The *hGH* protein is seen as a red precipitate.

Methods. Testes were fixed in Carnoy's, embedded in paraffin and cut into 5 μm sections. Sections were deparaffinized with xylene and re-hydrated using standard procedures. Indirect immunocytochemistry was performed using the reagents and suggested protocols of Vectastain ABC and Zymed SABC kits. The primary antibody, rabbit anti-*hGH*, was obtained from the National Hormone and Pituitary Program, Baltimore, Maryland, and used at either a dilution of 1/100 or 1/500. Aminoethyl carbazole (Zymed Lab. Inc.) was used as the substrate chromogen. Sections were counterstained with haematoxylin. Additional negative controls included the use of both the primary and secondary antibodies on testes isolated from nontransgenic animals. For the isolated epididymal sperm, transgenic animals were killed, the caput epididymis dissected out and cut into 8–10 pieces, and incubated for 30 min at 30°C in EKRB media²⁴. After 30 min the supernatant was removed, centrifuged for 5 min at 1,000 r.p.m., and the pellet resuspended in a small volume of EKRB media. A few μl of sample were spread onto a microscope slide, allowed to dry for about 1 min, and fixed in Carnoy's. After incubation in PBS for 30 min, immunocytochemical analysis was performed as described above.

suggesting that it is not incomplete sharing of post-meiotic gene products that leads to the immunonegative cells.

Development of the acrosome occurs adjacent to the nucleus during spermiogenesis^{9,11,12}. Juxtaposed to the acrosome is the cellular machinery responsible for synthesizing the acrosomal enzymes, the Golgi apparatus and the rough endoplasmic reticulum (RER)^{12,13}. Because *hGH* protein is localized to the

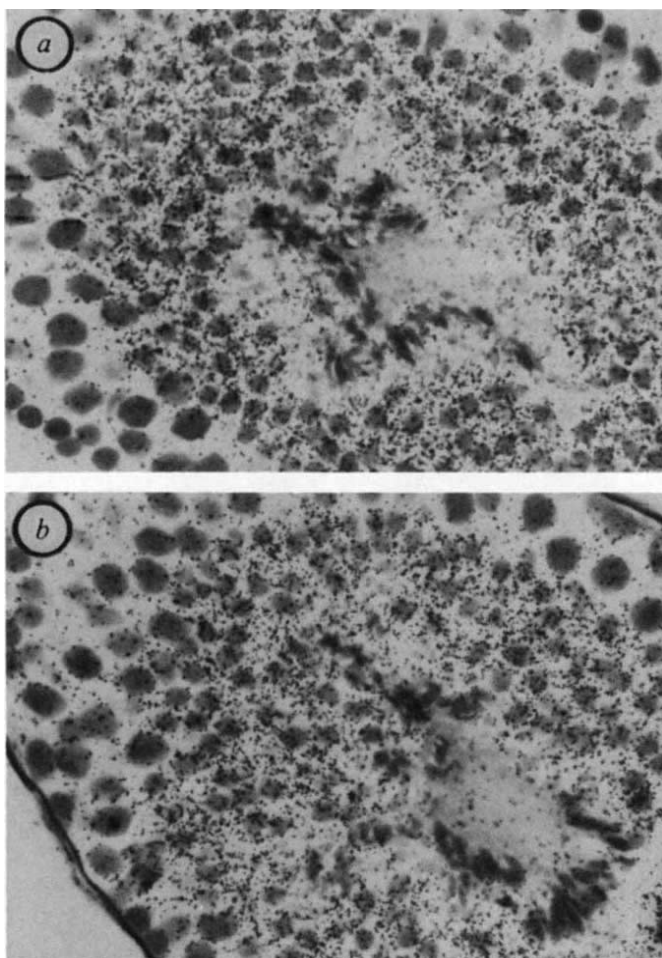


Fig. 4 *In situ* hybridization of testis sections hybridized with either an *hGH* antisense probe (a) or an *mPI* antisense probe (b). Sections were photographed at $\times 500$ magnification. See Fig. 2 legend for details of hybridization conditions.

acrosome, it is probably synthesized on the RER, at the site of acrosome formation, and is therefore not free in the cytoplasm¹⁴. We first detect *hGH* immunocytochemically in the developing acrosome (Fig. 3a). Although it is unlikely that vesicles bud off from the Golgi and move between the syncytial bridges, electron microscopy studies have shown that the endoplasmic reticulum does sometimes extend through the bridges¹⁵ suggesting that growth hormone protein could be traversing the bridges through the ER. Comparison of serial sections hybridized with *hGH*-specific and *mPI*-specific RNA probes (Fig. 4a, b), however, showed that within a seminiferous tubule there seemed to be an equal distribution of silver grains over all of the round spermatids regardless of the probe used. These results suggest that there could be movement of *mPI-hGH* mRNA, presumably through the intercellular syncytial bridges. Experiments designed to test unequivocally for movement of mRNA are currently in progress.

These results provide evidence against functional differences between sperm arising from post-meiotic gene expression. Our results are supported genetically, with one exception, by the absence of any cases of non-mendelian inheritance in the mouse¹⁶. The one exception involves preferential transmission of the *t* locus in male mice heterozygous for the *t* chromosome¹⁷. Male mice heterozygous for a complete *t*-haplotype can transmit the *t*-haplotype chromosome with a frequency as high as 99%¹⁸. Although transmission ratio distortion in *t*-haplotypes might be due to preferential retention of mRNA and/or protein in spermatids in which they are synthesized^{16,19}, it has yet to be demon-

strated that post-meiotic transcription is definitely involved in the transmission ratio distortion. Our data do, however, clearly show that, at least for the product of the *mPI-hGH* transgene, either mRNA and/or protein is free to move between the intercellular bridges which connect developing spermatids, and suggest that the products of most genes may be equally distributed among spermatids.

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Tnt1, a mobile retroviral-like transposable element of tobacco isolated by plant cell genetics

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Transposable elements can be identified by their ability to induce mutant alleles at new loci. The retrotransposon family is thought to transpose through an RNA intermediate and has many similarities to vertebrate proviruses^{1,2}. In plants, retrotransposons have been described in maize^{3,4}, *Arabidopsis*⁵ and wheat⁶, and non-viral retrotransposons in maize⁷. Most of these elements, however, have been found as non-mobile integrated units. Here, we report the isolation of the first tobacco (*Nicotiana tabacum*) transposable element, Tnt1, which seems to be the most complete mobile retrotransposon characterized in higher plants. Tnt1 has been isolated after its transposition into the nitrate reductase (NR) structural gene of tobacco, and transposition events have been detected through *in vitro* selection of spontaneous NR-deficient (NR⁻) mutant lines in cell cultures derived from tobacco mesophyll protoplasts. Tnt1 is 5,334 nucleotides long, contains two 610-base-pair-long terminal repeats and a single open reading frame of 3,984 nucleotides. Comparison of the Tnt1 open reading frame coding potential with those of the *Drosophila melanogaster copia*

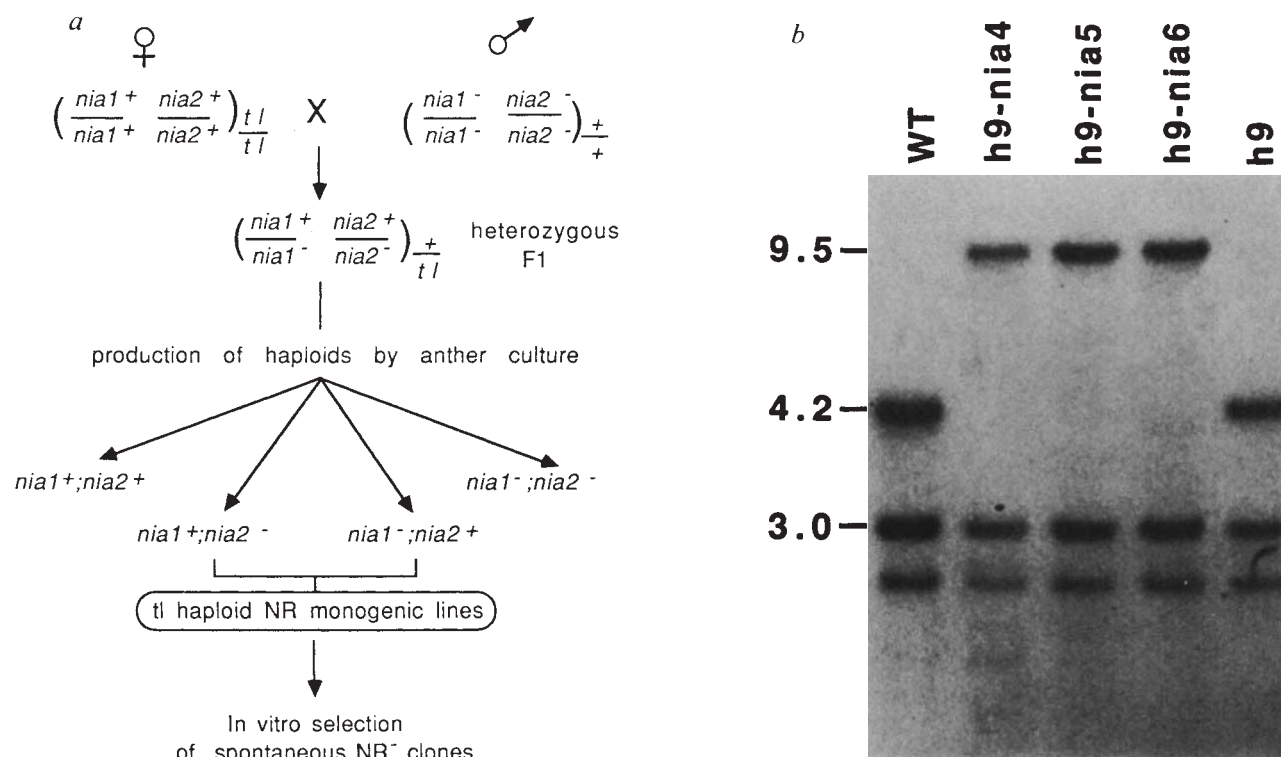


Fig. 1 Isolation and characterization of three spontaneous NR^- mutants induced by transposition of Tnt1 into the coding sequence of the nia2 locus. **a**, The construction of haploid monogenic lines from which spontaneous NR^- clones were selected. Stably mutated nia alleles from a NR^- ($\text{nia1}^-; \text{nia2}^-$) tobacco line¹² were introduced into a NR^+ ($\text{nia1}^+; \text{nia2}^+$) t1 line by crossing and subsequent anther culture²⁹ of the F1 . Haploid NR monogenic t1 lines ($\text{nia1}^+; \text{nia2}^-$) or ($\text{nia1}^-; \text{nia2}^+$) were screened by genetic tests on derived diploidized plants²⁹. Mesophyll protoplasts were isolated³⁰ from haploid lines and spontaneous chlorate-resistant clones were selected on derived colonies¹³. The NR deficiency of these clones was confirmed by their inability to grow on nitrate as the sole nitrogen source. NR^- plants were regenerated and propagated in the greenhouse by grafting³¹. **b**, Southern blot analysis of EcoRI restriction digests of total DNA isolated from three independent NR^- mutants selected in cell cultures from the h9 ($\text{nia1}^-; \text{nia2}^+$) t1 monogenic line. WT = wild-type XHFD8 tobacco line²⁹. Structure of the stably mutated nia genes originating from the NR^- line¹¹ is similar to the wild-type nia genes¹⁴. The probe was a 1.6 kb EcoRI NR cDNA fragment which hybridizes similarly to both alleles, revealing a 3 kb band from the wild-type nia1 gene and a 4.2 kb band from the wild-type nia2 gene. An additional 2.8 kb band shows homology to the probe, but is not correlated to the nia1 or nia2 structural genes¹⁴. In the three h9-Nia4 , h9-Nia5 and h9-Nia6 mutants, the nia2 4.2 kb EcoRI fragment has disappeared and a new 9.5 kb band is present. Sizes are in kb.

Methods. Total plant DNA was isolated by a modified Dellaporta procedure³² and purified on a CsCl gradient. DNA (5–6 μg per lane) was digested and Southern blot analysis was performed as previously described¹¹.

retrotransposon⁸, yeast Ty retrotransposon⁹, and vertebrate pro-retroviruses² revealed that Tnt1 is closely related to *copia* and carries all the functions known to be required for autonomous transposition by reverse transcription.

The primary aim of this work was the isolation of a transposable element thought to be responsible for a chlorophyllian somatic instability in a mutant tobacco line named t1 (ref. 10). We used cell genetic techniques to detect the transposition of this element into the NR structural gene, for which a cDNA molecular probe was available¹¹. Tobacco is an amphidiploid species arising from the combination of two diploid genomes of wild-type *Nicotianae* species. To induce nitrate reductase deficiency, two functional homoeologous genes (nia1 and nia2) encoding the apoenzyme of nitrate reductase have to be simultaneously mutated in the haploid tobacco genome¹². Haploid NR monogenic t1 lines were constructed, in which one of the two nia alleles is stably mutated, and in which a single transposition event can induce a NR^- phenotype (see Fig. 1a). Spontaneous NR^- clones were selected from mesophyll protoplast-derived cells by a direct selection for resistance to chlorate¹³. Several spontaneous NR^- clones were isolated at frequencies of up to 10^{-4} and regenerated into plants. Molecular analysis of regenerated NR^- plants showed that this phenotype was associated, in one-third of the clones analysed, with a

modification of the restriction pattern of the wild-type nia allele. In three independent NR^- mutants selected from the h9 NR monogenic line ($\text{nia1}^-; \text{nia2}^+$), the 4.2 kilobase (kb) EcoRI fragment present within the wild-type nia2 gene¹⁴ has disappeared and a new hybridizing band of about 9.5 kb is present (Fig. 1b). More detailed restriction mapping revealed that an insertion of 5.3 kb was located into the first exon of the nia2 gene in mutant h9-Nia6 , the second in mutant h9-Nia5 , and the third exon in mutant h9-Nia4 (data not shown).

To isolate the 9.5 kb EcoRI band, partial genomic libraries were constructed in phage λ NM1149 (ref. 15) from mutants h9-Nia4 and h9-Nia5 , and screened with the NR cDNA probe. Clone LNM94 was isolated from the h9-Nia4 library and clone LNM95 was isolated from the h9-Nia5 library. In both clones, a 5.3 kb insertion is found at the expected location in the nia2 gene. The structure of the nia regions adjacent to the insertion is similar to the structure of the wild-type gene, indicating that no other DNA rearrangement has occurred in the two mutants. Both insertions cross-hybridize and have similar restriction maps (Fig. 2), indicating that the NR^- phenotype is due to the transposition of the same mobile element into the nia2 gene. The element is, however, inserted in opposite orientation in each mutant. This transposable element has been named Tnt1 (for transposable element of *Nicotiana tabacum*).

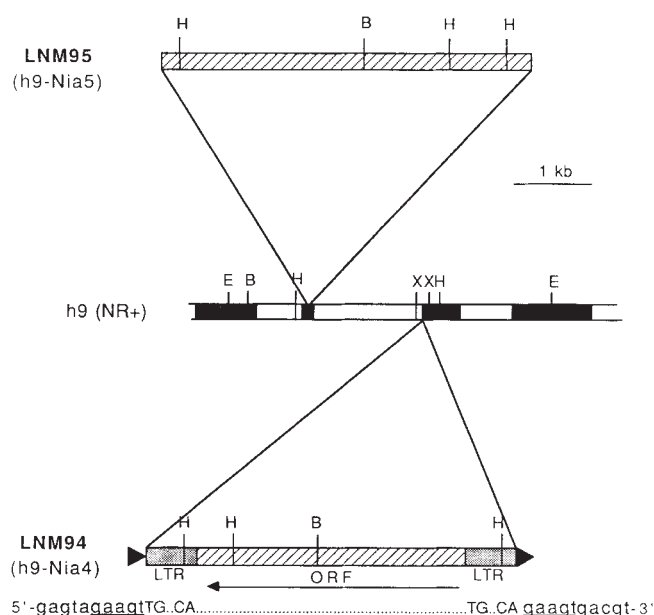


Fig. 2. Restriction maps of the two Tnt1 insertions found in clones LNM94 and LNM95. A restriction map of the NR⁺ (h9) *nla2* *Eco*RI fragment is shown for reference. Some structural features of the Tnt1 element of LNM94 are shown. Dark boxes represent the coding sequence of the *nla2* gene, hatched boxes the Tnt1 insertion, and shaded boxes the LTRs. Arrow indicates the Tnt1 ORF orientation. The sequence of LTR terminal inverted repeats is represented in capital letters, the sequence of the *nla2* gene¹⁴ in lower-case letters, and the sequence of the target site duplications (dark triangles) is underlined. B = *Bam*HI, E = *Eco*RI, H = *Hind*III and X = *Xho*I.

Methods. Plant DNA (150 µg) was digested by *Eco*RI and fractionated on sucrose gradient as described³³. Fractions containing the 9.5 kb band hybridizing to the NR cDNA probe were purified and ligated to the insertion vector λ NM1149 (ref. 15). Because of the high instability of the Tnt1 insertion, further purifications of positive recombinant clones, and subcloning in pBluescript (Stratagene) were performed on recA⁻ host strains.

The complete sequence of the Tnt1 element found in the mutant h9-Nia4 was determined by the dideoxynucleotide method¹⁶. Tnt1 is 5,334 base pairs (bp) long and is flanked by a duplication of a short region of host DNA (5 bp) at the integration site. Tnt1 has structures typical of transposable elements of the retrotransposon family (Fig. 2). It is bounded by two identical long terminal direct repeats (LTRs) of 610 bp, which are terminated by the consensus sequence 5'TG...CA-3'. Moreover, Tnt1 internal features include most of the structural and functional regions commonly found in retrotransposons and retroviruses. Sequence analysis of the 4,115 bp internal domain of Tnt1 has revealed a single large open reading frame (ORF) of 1,328 amino acids, oriented in the opposite direction to the *nla* coding sequence in the h9-Nia4 mutant. The putative protein encoded by the Tnt1 ORF was compared with retroviral gene products¹⁷⁻¹⁹ and the putative protein encoded by *cop*ia ORF⁸. The general organization of the Tnt1 ORF is very similar to the one in *cop*ia, both in overall structure (*gag-pol*) and in length (1,328 amino acids in Tnt1, 1,409 in *cop*ia). At the amino-acid level also, a high degree of homology was found between the putative polyproteins encoded by the ORFs of these two elements (Fig. 3): 29% homology for the *gag* polyprotein product (residues 1-265), 42% homology for the endonuclease domain of the *pol* polyprotein product (residues 420-720), and 38% homology for the reverse transcriptase domain of the *pol* polyprotein product (residues 810-1,328 for Tnt1 and 890-1,404 for *cop*ia). Similarities include several short stretches of amino acids that are highly conserved in all proutetroviruses and retro-

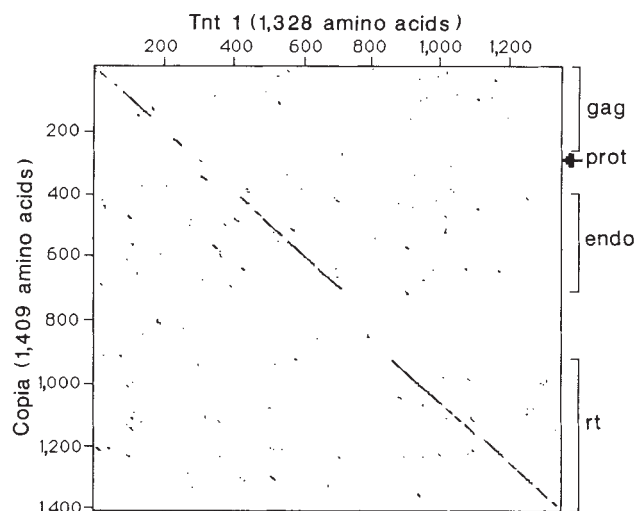


Fig. 3 Homology matrix comparisons between amino-acid sequences of the Tnt1 and the *cop*ia ORFs. A computer program³⁴ was used to generate diagonal lines indicating segments of 20 residues that show similarity above a threshold level. Residue numbers are indicated. The approximate regions corresponding to *gag*, and the endonuclease (*endo*) and reverse transcriptase (*rt*) domains of the *pol* gene are shown. The active site of the protease (*prot*) is indicated by an arrow. Respective positioning of all these regions are similar in Tnt1 and *cop*ia. In proutetroviruses and retroviral-like elements, the *gag* gene encodes for the structural core proteins, and the polyprotein encoded by the *pol* gene is matured by cleavage in several functional units, which are mainly the protease, the endonuclease and the reverse transcriptase. No homology to the *env* region of retroviruses has been found in *cop*ia and Tnt1.

Methods. Average score values were calculated for pairs of segments of 20 residues, whose amino-acid sequences were compared between Tnt1 and *cop*ia. If the average score value is greater than 238, a diagonal line is generated on the corresponding position of the homology matrix. This threshold value is the degree of similarity that would occur by chance with a probability of less than 2×10^{-4} . All the computer analyses were performed using the programs available from the Centre Interuniversitaire de traitement de l'Information (CITI 2, Faculté de Médecine, Paris).

transposons, which are summarized in Fig. 4. The *gag* regions of *cop*ia and Tnt1 contain a 14-amino-acid domain located at exactly the same position in both ORFs (amino acids 232-245), which is highly conserved among retroviral nucleic acid-binding proteins²⁰. These proteins are required for accurate positioning of the tRNA primer on the replication initiation site at the 5' end of the RNA genome²¹. In most retroviruses and retrotransposons, the 5' end of the *pol* gene codes for a protease involved in the processing of polyproteins. The conserved sequence Asp-Thr(Ser)-Gly is replaced in Tnt1 by the Asp-Thr-Ala sequence, which presumably could also act as an active site of the protease²². Moreover, this sequence is found at comparable locations in Rous sarcoma virus (RSV), *cop*ia and Tnt1 elements, and the relative spacing between this site and the nucleic acid-binding domain is similar in the three genomes. Regions of highest homology between the Tnt1 ORF and *cop*ia also include conserved domains identified among a wide variety of reverse transcriptase and polymerase gene products²³⁻²⁵. One of the most highly conserved domains was found at amino acids 1,005-1,014 of the Tnt1 ORF (amino acids 1,012-1,021 of the *cop*ia ORF). This region (Tyr-Met or Val-Asp-Asp followed by strictly hydrophobic long-chain amino acids) is found not only in reverse transcriptases, but also in RNA-templated viruses²⁶. The 3' region of the *pol* gene of a variety of vertebrate retroviruses also encodes an endonuclease^{1,23}. In Ty and *cop*ia, there is homology to this region at the 5' end of the *pol* gene⁸, as in Tnt1.

Fig. 4 Comparison of the Tnt1 ORF with conserved domains found in retroviruses and retrotransposons. Amino-acid sequences are indicated in capital letters, and the corresponding nucleotide sequence for Tnt1 is indicated in lower-case letters. Conserved and nearly invariant amino acids are shaded. Fully conserved positions are indicated by arrows. *Copia* is a retrotransposon from *Drosophila melanogaster*⁸; Ty-912 is a member of the retrotransposon family Ty1 in *Saccharomyces cerevisiae*⁹; MMULV is the Moloney murine leukemia virus¹⁷; RSV is the Rous sarcoma virus¹⁸; HIV-1 is the human immunodeficiency virus¹⁹. Alignments are those previously published²³⁻²⁵. Nucleic acid-binding domain²⁰; the cysteine and histidine residues involved in the zinc-binding domain are boxed. Protease domain²²; the conserved D-T-(S)-G amino-acid sequence is boxed and numbers indicate amino-acid positions, starting from gag (Tnt1, *copia* and RSV), pol (MMULV, HIV-1) or ORFb (Ty-912). In the endonuclease domain²³, only the most highly conserved domain is represented. In the reverse transcriptase domain, the three motifs are compared as in ref. 25 and the length separating the motifs is indicated between brackets.

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Note added in proof: A quick analysis of the amino-acid sequence homologies between Tnt1 and the Ta1 retrotransposon recently isolated in *Arabidopsis*⁵ has given the following approximations: endonuclease (integrase) domain, 42%; reverse transcriptase domain, 52% (D. Voytas, personal communication). Both elements seem therefore to be more closely related to each other than to *copia*.

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The relationship of a prochlorophyte *Prochlorothrix hollandica* to green chloroplasts

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It is generally accepted that chloroplasts arose from one or more endosymbiotic events between an ancestral cyanobacterium and a eukaryote¹. Such an origin fits well in the case of the chloroplasts of rhodophytes that, like cyanobacteria, contain chlorophyll *a* and phycobilin pigments². The green chloroplasts from higher plants, green algae, and euglenoids however, contain chlorophyll *b* as well as chlorophyll *a*, and lack phycobilins. Consequently, it has been suggested that they arose independently of the rhodophyte chloroplasts, from an ancestral prokaryote containing that complement of pigments³. The 'prochlorophytes' *Prochloron didemni* (an exosymbiont on didemnid ascidians^{4,5}) and *Prochlorothrix hollandica* (a recently discovered, free-living, filamentous form⁶) have been suggested to be modern counterparts of the ancestor of the green chloroplasts because they are prokaryotes that also contain both chlorophylls *a* and *b*, and lack phycobilins^{7,8}. We report here a 16S rRNA-based phylogenetic analysis of *P. hollandica*. The organism is found to fall within the cyanobacterial line of descent, as do the green chloroplasts, but it is not a specific relative of green chloroplasts. Thus, similar pigment compositions do not necessarily reflect close evolutionary relationships.

Analysis of RNase T1-generated oligonucleotide catalogues of 16S rRNA from several samples of *Prochloron* sp. indicated that this symbiont falls within the cyanobacterial line of descent, but it seemed to be more closely related to the filamentous, heterocystous cyanobacteria of the genera *Nostoc* and *Fis-*

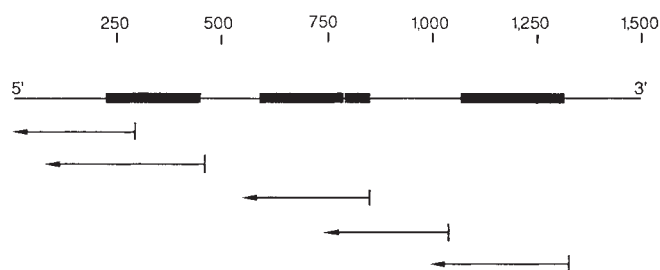


Fig. 1 Regions of 16S rRNA sequenced. *Prochlorothrix hollandica* cells were grown in liquid batch culture using medium BG-11²⁶. Ribosomal RNA was obtained and purified as described¹³, except that after phenol extraction and ethanol precipitation of the cell lysate, large rRNA was purified by electrophoresis on a denaturing 2.5% polyacrylamide gel. The band containing 16S rRNA was excised from the gel and the RNA was electroeluted into 3M NaOAc (pH 5.5), followed by two rounds of ethanol precipitation. Dideoxynucleotide-terminated sequencing of 16S rRNA using reverse transcriptase and synthetic oligodeoxynucleotide primers was as described^{13,27}. The *P. hollandica* 16S rRNA is represented as a line segment about 1,500 nucleotides in length. Arrows designate those regions of sequence determined from the use of individual oligodeoxynucleotide primers. Thickened regions of the line segment represent the nucleotides used in the sequence analysis.

cherella than to green chloroplasts^{9,10}. This interpretation of the data has been disputed for methodological reasons¹¹. Although the inability to cultivate *Prochloron* sp.¹² has retarded the investigation of its biochemistry and molecular biology, the discovery of *P. hollandica*⁶ provided a tractable model for detailed investigations of prochlorophytes. The presence of chlorophyll *b* in *P. hollandica* suggests that it might be the closest known, free-living relative to the green chloroplasts.

At present, the best method for evaluating evolutionary relationships is the comparison of homologous macromolecular sequences. The 16S rRNA was the natural choice for the phylogenetic analysis of *P. hollandica* because of the broad database available, of cyanobacterial and chloroplast 16S rRNA sequences¹³. About 1,200 nucleotides of the *P. hollandica* 16S rRNA sequence were determined by dideoxynucleotide termina-

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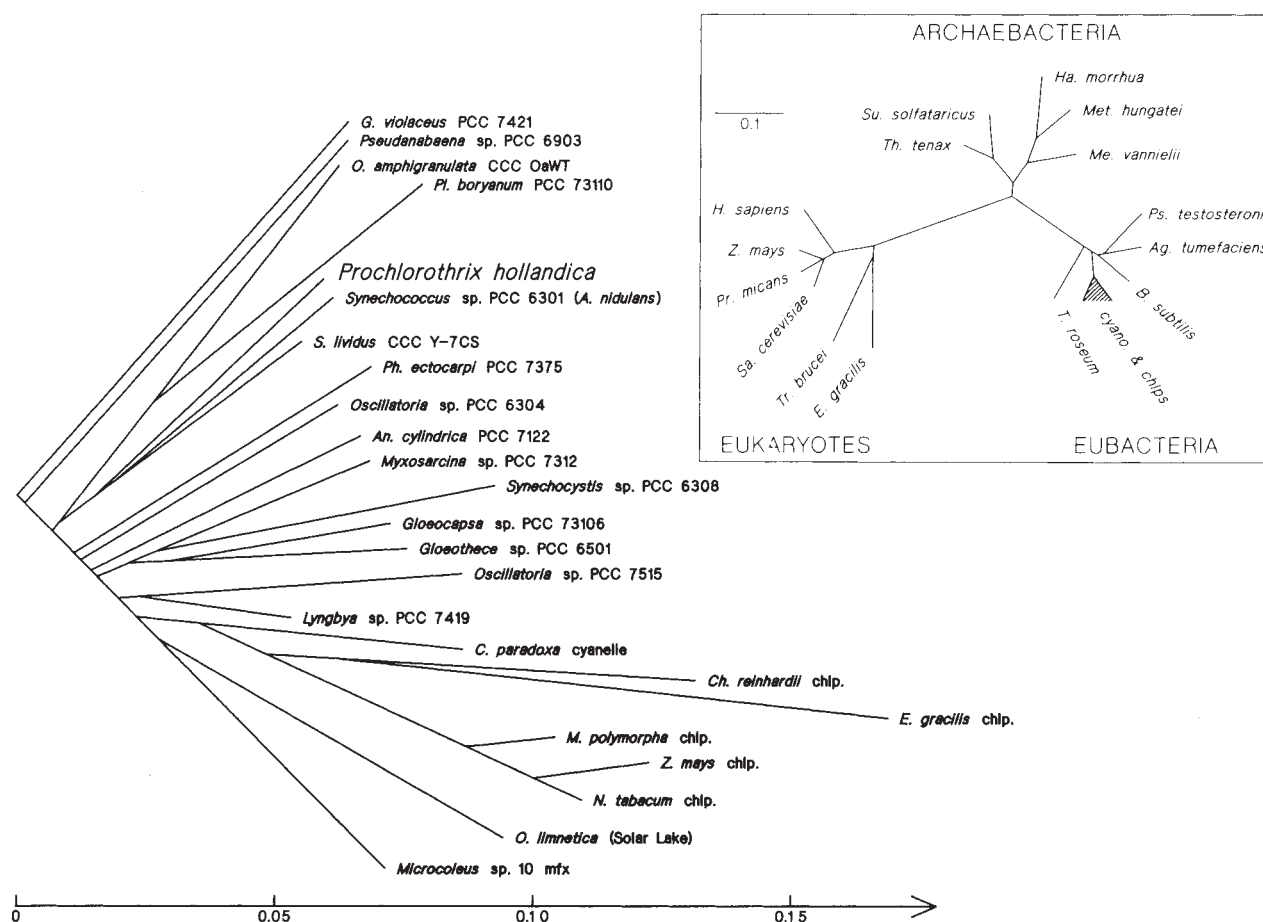


Fig. 2 Rooted phylogenetic tree depicting evolutionary relationships among 16S rRNAs from *P. hollandica*, cyanobacteria, green chloroplasts, and a cyanelle. 16S rRNA sequences from the outgroup organisms *Agrobacterium tumefaciens*, *Bacillus subtilis* and *Pseudomonas testosteroni* were used to locate the root¹³. In this representation, evolutionary distances are proportional to only the horizontal component of the segment lengths. The scale is in units of fixed point mutations per sequence position. Sequence analysis was carried out by a distance matrix method^{13,17,28}. Inset: unrooted phylogenetic tree depicting the three primary kingdoms and the relation of the cyanobacteria and chloroplasts to some other eubacteria. Segment lengths are proportional to evolutionary distances. The scale bar represents 0.1 fixed point mutations per sequence position. Adapted from ref. 13. Abbreviations: A., *Anacystis*; Ag., *Agrobacterium*; An., *Anabaena*; B., *Bacillus*; C., *Cyanophora*; CCC, Castenholz Culture Collection; Ch., *Chlamydomonas*; chl(s), chloroplast(s); cyano., cyanobacteria; E., *Euglena*; G., *Gloeobacter*; H., *Homo*; Ha., *Halococcus*; M., *Marchantia*; Me., *Methanococcus*; Met., *Methanospirillum*; N., *Nicotiana*; O., *Oscillatoria*; PCC, Pasteur Culture Collection; Ph., *Phormidium*; Pl., *Plectonema*; Pr., *Prorocentrum*; Ps., *Pseudomonas*; S., *Synechococcus*; Sa., *Saccharomyces*; Su., *Sulfolobus*; T., *Thermomicrobium*; Th., *Thermoproteus*; Tr., *Trypanosoma*; Z., *Zea*.

tion, using reverse transcriptase elongation from oligodeoxynucleotide primers on purified 16S rRNA (Fig. 1). The sequence has been deposited with GenBank and is available from the authors upon request. The inference of the evolutionary relationship of *P. hollandica* to other organisms used a distance matrix method to analyse 677 sequence positions that are approximately equally distributed among the three major domains of the 16S rRNA secondary structure¹⁴ and are unequivocally homologous in all the organisms. The results are presented as a phylogenetic tree in Fig. 2.

P. hollandica is a deeply branching member of the cyanobacteria, most closely related to two unicellular forms, *Synechococcus* sp. strain PCC 6301 (*Anacystis nidulans*) and *Synechococcus* 'lividus' strain CCC Y7C-S. *P. hollandica* is not specifically related, to the exclusion of other cyanobacteria, to the coherent phylogenetic grouping of the green chloroplasts and the cyanelle of *Cyanophora paradoxa*¹³. We feel that the continuous sequences of the cyanobacterial 16S rRNAs¹³ do not overlap sufficiently with the RNase T1-generated, 16S rRNA oligonucleotide catalogues of *Prochloron didemni*⁹ and the chloroplast of the red alga *Porphyridium* sp.¹⁵ to allow the reliable inference of a phylogenetic tree that includes these latter components.

The assumption that all sequence positions are equally subject

to change can lead to significant errors in the estimation of sequence divergence¹⁶. Although the likelihood of such errors decreases when sequences of closely related organisms are compared, the possibility of such an occurrence in our analysis was checked by inferring phylogenetic trees under the assumption that the rates of change of sequence positions are described by a log-normal distribution¹⁷. No differences in tree topology were found.

The reliabilities of several features of the inferred phylogeny were examined by a 'bootstrap' analysis of the data in which sequence positions were randomly selected, with replacement, to produce data sets that were analysed in the same manner as the original data set¹⁸. Twenty-five 'bootstrapped' phylogenetic trees were generated for this analysis. In 19 of them (76%), *P. hollandica* formed a coherent subgroup with *Synechococcus* sp. strain PCC 6301 and/or *Synechococcus* 'lividus' strain CCC Y7C-S to the exclusion of all other members of the tree. In 23 'bootstrapped' trees (92%), *P. hollandica* formed coherent subgroups with one or both *Synechococcus* spp. and/or *Phormidium ectocarpi* strain PCC 7375. The association of the cyanelle of *Cyanophora paradoxa* with the green chloroplasts to form a coherent, exclusionary subgroup occurred in 22 'bootstrapped' trees (88%). In no instance did *P. hollandica* group

with one or more of the green chloroplasts or cyanelle to the exclusion of other organisms in the tree. This analysis firmly supports the conclusion that *P. hollandica* is not a specific relative of the green chloroplasts.

On the basis of these rRNA sequence comparisons, the closest known relative of green chloroplasts is the cyanelle of *C. paradoxa*. Cyanelles are believed to be the result of a relatively recent endosymbiosis between a cyanobacterium and a eukaryote¹⁹. This hypothesis is supported by the fact that cyanelles have both cyanobacterial and chloroplast-like features. Cyanobacterial qualities of cyanelles include a rudimentary peptidoglycan wall, and the presence of chlorophyll *a* and phycobilins. Cyanelles lack chlorophyll *b* and the membrane arrangements that are characteristic of green chloroplasts¹⁹. The chloroplast-like features of cyanelles include small genome size and genome organization^{20,21}. Furthermore, as with chloroplasts, the expression of many cyanelle proteins is under the control of the eukaryotic host²².

Some lineages that are not known to produce chlorophyll *b* diverged from the main cyanobacterial line at positions intermediate between the lines leading to the green chloroplasts and to *P. hollandica* (Fig. 2). This result implies that the ability to synthesize chlorophyll *b* arose independently in the ancestors of *P. hollandica* and the green chloroplasts, or that it was acquired by lateral transfer. Chlorophylls *a* and *b* differ in a minor way: the oxidation state of a single carbon (methyl in chlorophyll *a* but aldehyde in chlorophyll *b*) so the acquisition of the ability to synthesize chlorophyll *b* would not seem to be a significant biochemical change. A less likely interpretation of the phylogenetic relationships is that *P. hollandica* and the progenitor(s) of green chloroplasts shared an ancestor that produced chlorophyll *b*, but that multiple losses of the ability to synthesize chlorophyll *b* occurred among the intermediate cyanobacterial sublines. In any case, however, we must conclude that similar pigment compositions do not necessarily reflect particularly close evolutionary relationships.

All the currently available data indicate that cyanobacteria, prochlorophytes, and chloroplasts constitute a 'holophyletic' group; that is, a group that consists of a common ancestor and all lineages derived from it²³. The creation of a new taxon, the Prochlorophyta, has been recommended to accommodate prokaryotes that contain chlorophylls *a* and *b* and lack phycobilins, to the exclusion of the cyanobacteria²⁴. Such a taxon has been previously criticized on the grounds that pigment composition does not constitute sufficient evidence for the creation of a new taxon²⁵. This report, that the ability to synthesize chlorophyll *b* occurs sporadically in separate lineages, shows that such a taxon would not constitute a natural grouping. It would be a 'polyphyletic' group, one composed of selected members of independent lineages.

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***psbA* genes indicate common ancestry of prochlorophytes and chloroplasts**

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It has long been suspected that chloroplasts evolved after an endosymbiotic event involving a photosynthetic prokaryote, presumably a cyanobacterium, and a eukaryotic organism. Recent studies have provided strong evidence about the cyanobacterial nature of chloroplasts¹. Since the discovery of prochlorophytes², oxygen-evolving photosynthetic prokaryotes containing chlorophyll *a* and chlorophyll *b* and lacking phycobiliproteins, there has been speculation that these represent evolutionary intermediates between cyanobacteria and chloroplasts³. *Prochloron* sp., the first described prochlorophyte, proved difficult to work with because it is an obligate symbiont of marine ascidians³. *Prochlorothrix hollandica*, a recently isolated, freshwater filamentous prochlorophyte, is easily maintained in the laboratory^{3,4}. Overall pigment composition and thylakoid membrane structure of *P. hollandica* suggest it has intermediate characteristics between cyanobacteria and the chloroplasts of higher plants³. The *P. hollandica psbA* genes, which encode the photosystem II thylakoid protein D1, were cloned and sequenced and the sequences compared to those reported for cyanobacteria, a green alga, a liverwort, and several higher plants. The two *psbA* genes present in *P. hollandica* encode an identical amino-acid sequence. As in all chloroplast *psbA* genes, there is a seven amino-acid gap near the C terminus of the derived protein relative to the protein predicted by cyanobacterial genes⁵, suggesting that *P. hollandica* is part of the lineage that led to chloroplasts after a divergence from cyanobacteria. This hypothesis is also supported by phylogenetic analysis of derived D1 amino-acid sequences from *psbA* genes of thirteen taxa on the basis of parsimony.

Heterologous *psbA* gene probes from *Synechococcus* sp. strain PCC 7942 (ref. 6) were used to identify regions of sequence similarity in *P. hollandica*. These probes hybridized to two fragments when DNA was digested with either *EcoRI* (6.4 and 5.35 kilobases kb) or *BamHI* (3.0 and 2.9 kb). Both *EcoRI* fragments were cloned and nucleotide sequence analysis revealed that each clone contained an entire *psbA* open reading frame (Fig. 1). The genes were named such that *psbAI* was sequenced from the 6.4 kb *EcoRI* fragment (which includes the 3.0 kb *BamHI* fragment) and *psbAII* was sequenced from the 5.35 kb *EcoRI* fragment (which includes the 2.9 kb *BamHI* fragment). Only two of the 1,059 base pairs (bp) in the open reading frame differed between the nucleotide sequences of the two genes. These differences represent silent transition mutations which did not alter the predicted polypeptide sequences encoded by these genes even though one, nucleotide 1,027, occurred in the first base position of a codon. The number of genes present and the nature of the predicted polypeptide is in contrast to the situation in both cyanobacterial and chloroplast genomes. All

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Fig. 1 Nucleotide and derived amino-acid sequences of the two *psbA* genes from *P. hollandica*. The nucleotide sequences upstream and downstream of the 1,059 bp open reading frame (ORF) are shown for *psbAI* and *psbAII*. Regions of sequence identity in the upstream and downstream regions of *psbAI* and *psbAII* are underscored. The nucleotide sequence of the *psbAII* ORF is shown in its entirety. Differences in the *psbAI* sequence are denoted above the *psbAII* sequence at positions 282 and 1,027. The single predicted amino-acid sequence for the protein encoded by both *psbAI* and *psbAII* is listed below each corresponding line of nucleotide sequence.

Methods. DNA was digested with *EcoRI*, ligated to *EcoRI*-cleaved λ GT-10 cloning vector, and packaged *in vitro*. The library was plated on *Escherichia coli* host strain ER1458 (*mcr*⁻) and the resulting plaques were transferred to DuPont colony/plaque screen and probed with nick-translated¹⁴ *psbA*-containing fragments from *Synechococcus* sp. PCC 7942. Positively identified plaques were picked, the phage amplified, and DNA isolated¹⁴. Cloned DNA was subcloned into plasmids for sequencing using a Sequenase dideoxynucleotide kit (United States Biochemical Corporation) and reactions were separated on a 7 M urea, 6% polyacrylamide gel¹⁴. Nucleotide sequences of different regions of both *psbAI* and *psbAII* were determined by a combination of subcloning restriction fragments, making nested deletions with exonuclease III, and preparing synthetic oligonucleotides from known regions of the genes to use as primers. Sequencing reactions were completed for each gene from overlapping clones to account for all bases on both DNA strands.

-80			
I	CTATAGTTGG	GTACAGTTCA	AAGACGGTTA TCCGCACCGA ACGGAAATTT CATACCTCTGT ACCTTGGCAAT CATATAATACC
II	AAAGTTCACT	ATACCGGTAC	TATGAGTTCC AACGGTTCAA AAAAAACCGC CTGTACATAG AAAACGAAAT CATATAATACC
		66	
II	ATG ACA ACC	GCA CTT CGC	CAG CGT GAA AGC GCT AAT GCG TGG GAA CAG TTT TGC CAG TGG ATC GCC
		132	
II	AGC ACC	GAG AAC	CGC CTG TAT GTG GGT TGG TTC GGC GTG ATC ATG ATC CCC ACC CTG CTG ACT GCC
		198	
II	ACC ATC	TGC TTC	ATC ATC GCC TTC ATC GCT GCT CCC CCC GTG GAC ATC GAC GGT ATC CGT GAG CCT
		264	
II	GTT GCT	GGT TCC	TTG ATG TAC GGC AAC AAC ATC ATC TCC GGT GCT GTG GTT CCC TCC TCC AAC GCG
		330	
I	ATC GGT	CTG CAC	TTC TAC CCC ATT TGG GAA GCA GCC AGC ATG GAC GAG TGG CTC TAC AAC GGT GGT
		396	
II	CCT TAC	CAG TTG	GTG GTG TTC CAC TTC CTG ATC GGC ATC TTC TGC TAC ATG GGT CGT GAG TGG GAA
		462	
II	CTC TCC	TAC CGC	CTT GGG ATC CGT CCC TGG ATC TGT GTC GGT TAC TCC GCT CCT GTG GCC GCT GCG
		528	
II	ACC GCT	GTG TTC	TTG ATC TAC CCC TTG GGC CAA GGT TCC TTC TCT GAT GGA ATG CCC CTG GGT ATC
		594	
II	TCT GGT	ACC TTC	AAC TTC ATG TTG GTC TTC CAG GCT GAG CAC AAC ATC CTG ATG CAC CCC TTC CAC
		660	
II	ATG CTC	GGT GTG	GCT GGT GTC TTC GGT GGT TCC TTG TTC TCC GCC ATG CAC GGT TCT TTG GTC ACC
		726	
II	TCC TCC	TTG GTT	CGT GAA ACC TCT GAG AAC GAG TCC CAG AAC TAC GGC TAC AAG TTC GGT CAA GAA
		792	
II	GAA GAG	ACC TAC	AAC ATC GTG GGT GCC CAC GGT TAC TTC GGT CGC CTG ATC TTC CAA TAT GCT TCT
		858	
II	TTC AAC	AAC AGC	CGT GCC CTG CAC TTC TTC CTC GGT GGT TGG CCC GTT GTC GGC ATC TGG TTT ACC
		924	
II	TCT CTG	GGT ATC	TCC ACC ATG GCC TTC AAC CTC AAC GGT TTC AAC TTC AAC CAG TCC GTG ATG GAC
		990	
II	AGC CAA	GGT CGC	GTT ATC AGC ACC TGG GCC GAC ATC CTC AAC CGT GCC AAC CTC GGT TTT GAA GTC
		1056	
I	ATG CAC	GAG CGC	AAC GCT CAC AAC TTC CCC CTT GAC CTG GGT GGT GTC AAA GCT CCT TCC ATC ATC
		1132	
I	GGT TAA	GTTCAGTTGC	TCTACTCATC TCCTAGCCTA GGCTAGACCT AAAGCGTCTC TTGGGGGGGC GTTTTGGCT
II	GGT TAA	GTTCAGTTGC	ACTACTCATC TAATAGCCTA GGCTAGACCT AAAGCGTCTC CTTTCGGGGG GCGGCTTTT
		G *	

cyanobacteria that have been examined have three or more copies of *psbA* in their genomes which encode two different polypeptides^{5,6} whereas the small circular chromosome of most chloroplasts contains a single copy of *psbA*⁷.

The predicted D1 polypeptide of cyanobacteria contains 360 amino acids compared to 353 amino acids in *P. hollandica* and higher plants (352 amino acids in *Chlamydomonas*). Figure 2 shows a comparison of the C-terminal region of the D1 protein predicted from *psbA* genes from various cyanobacteria, eukaryotic species, and *P. hollandica*. Plastid and *P. hollandica* polypeptide sequences all share a seven amino-acid gap relative to the cyanobacterial sequences at a position six amino acids from the end of the polypeptide. The presence of well-documented insertion/deletion events in bacterial genomes has been implicated as a more reliable indicator of phylogenetic relationships than nucleotide or amino-acid sequences⁸: the definitive gap among the *psbA* genes of specific lineages suggests a more recent common ancestor among green plant chloroplasts and *P. hollandica* than among chloroplasts and cyanobacteria.

Cyanophora paradoxa (a photosynthetic protozoan) has been implicated as a descendant of the progenitor to green chloroplast-bearing organisms because of the presence of phycobiliproteins, rather than chlorophyll *b*, in the chloroplast-like cyanelle⁹. Comparisons of 16S rRNA sequences, however, show that *C. paradoxa* is not directly related to plastids and probably

represents an independent endosymbiotic event¹. They also suggest that a cyanobacterium, possibly similar to the endosymbiont of *C. paradoxa*, was probably the progenitor of chloroplasts¹; however, a prochlorophyte was not included in that study. The *psbA* gene of *C. paradoxa* has recently been sequenced (W. Löffelhardt, personal communication). The seven amino acids characteristic of cyanobacterial sequences, but lacking in chloroplast-containing organisms, are present in the sequence of *C. paradoxa*. This strengthens the correlation between phycobiliprotein-containing organisms having a D1 protein of 360 amino-acid residues and chlorophyll *b*-containing organisms lacking the seven amino-acid domain at the C terminus of the protein. That *P. hollandica* also lacks this domain is further evidence that prochlorophytes, *P. hollandica* in particular, are members of the same lineage as the endosymbiont chloroplast progenitor which diverged from cyanobacteria. If prochlorophytes are not of this lineage, both chlorophyll *b* and *psbA* sequences shortened at the same position were derived independently in the two lineages.

As a further test to determine the relationships of *P. hollandica* to cyanobacteria and green chloroplast-containing organisms, the predicted polypeptide sequences of *psbA* genes from several species of vascular plants, a non-vascular plant, a green alga, four cyanobacteria, and *P. hollandica* were compared using PAUP, a computer program designed to determine phylogenetic

<i>Anabaena</i> 7120	(1) LDLA AGEVAPVALTAPAING*
	(2) LDLA AGEVAPVAISAPAING*
<i>Fremyella diplosiphon</i>	LDLA AGEVAPVALTAPAING*
<i>Synechococcus</i> 7942	(1) LDLA AGEATPVALTAPSIHG*
	(2) LDLA AGEATPVALTAPAING*
<i>Synechocystis</i> 6803	LDLA SGDAQMVVALNAPAIEG*
<i>Chlamydomonas</i>	LDLA STN-----SSNN--*
<i>Marchantia polymorpha</i>	LDLA AVE-----APAVNG*
Spinach	LDLA AIE-----APSTNG*
Soybean	LDLA AID-----APSING*
Alfalfa	LDLA AVE-----APSING*
<i>Prochlorothrix hollandica</i>	LDLA AVK-----APSIIG*

Fig. 2 Comparison of amino-acid sequences at the C terminus of the D1 polypeptide from different genera: *Anabaena*⁵, *Fremyella diplosiphon*¹⁵, *Synechococcus*⁶, *Synechocystis*¹⁶, *Chlamydomonas*¹⁷, *Marchantia polymorpha*¹⁸, spinach¹⁹, soybean²⁰, alfalfa²¹, *Prochlorothrix hollandica*. The C-terminal amino acids from residue 341 to the termination codon (*) are shown for each sequence, and those conserved among all sequences analysed are in bold type. Numerals following the genera *Anabaena*, *Synechococcus*, and *Synechocystis* refer to strain designations of the Pasteur Culture Collection. The *psbA* gene families of *Anabaena* sp. strain 7120 and *Synechococcus* sp. strain 7942 encode two slightly different D1 proteins; in each case the product of *psbA1* is designated by (1) and the product of the other members of the gene family is designated (2). Other plants with sequences identical to that of spinach in this region are *Amaranthus hybridus*²², *Nicotiana debneyi*¹⁹, *N. tabacum*²³, *Petunia hybrida*²⁴, and *Solanum nigrum*²⁵.

relationships based on parsimony¹⁰. Sequences of all taxa were aligned and were analysed by treating the gap region in four different ways: (1) excluding amino acids at positions 348–354 of cyanobacterial sequences from the analysis to compare the 353 remaining amino acids; (2) treating the seven amino-acid positions in the gap region as a single binary character (that is, presence or absence of this domain); (3) weighting each position of the gap region by 0.14 so the entire region is equal to a single character change with no loss of information at each position; (4) considering each position of the gap region as a separate and unique character. When amino acids of the gap region were completely excluded from the analysis (treatment 1), 30 equally parsimonious trees were produced, 15 of which were similar to that shown in Fig. 3 and 15 that associated *P. hollandica* in a lineage with the two *Synechococcus* sequences. The additional trees within each of these are accounted for by polychotomies and minor differences in topology of branches leading to higher plants. When the gap region was included in the analysis in any way (treatments 2, 3 or 4), tree topology was the same as in Fig. 3 with the major difference among the four treatments being the length of the branch (labelled *a*) leading to *P. hollandica* and chloroplast lineages: *a* was shortest in treatment 1 and longest in treatment 4 (data not shown). The length of each branch on the tree reflects the number of character state differences unique to that branch and the amount of divergence from a most recent common ancestor. The phylogenetic trees produced thus corroborate the phylogeny inferred from the gap distribution which places *P. hollandica* closer than cyanobacteria to green plant chloroplasts.

The predicted rate of divergence among highly conserved genes in bacteria has been estimated to be approximately 1% per 50 million years (Myr)^{11,12}. The percentage similarity among *P. hollandica* *psbA* nucleotide sequences and those of *Anabaena* or *Synechococcus* range from 81.6% to 83.6% for the six genes examined, which would place the time of divergence between the cyanobacterial and prochlorophyte lines at about 900 Myr ago. This figure is within the range (800–2,000 Myr ago) previously predicted for the time of divergence between plastids and cyanobacteria based on 16S rRNA comparisons^{11,12}.

With the discovery of a third member of the *Prochlorophyta* which occupies deep marine euphotic zones¹³, studies on this interesting group should continue to contribute to our understanding of the phylogeny of chloroplasts. Because *P. hollandica* is easily grown in culture^{3,4} and represents an organism that

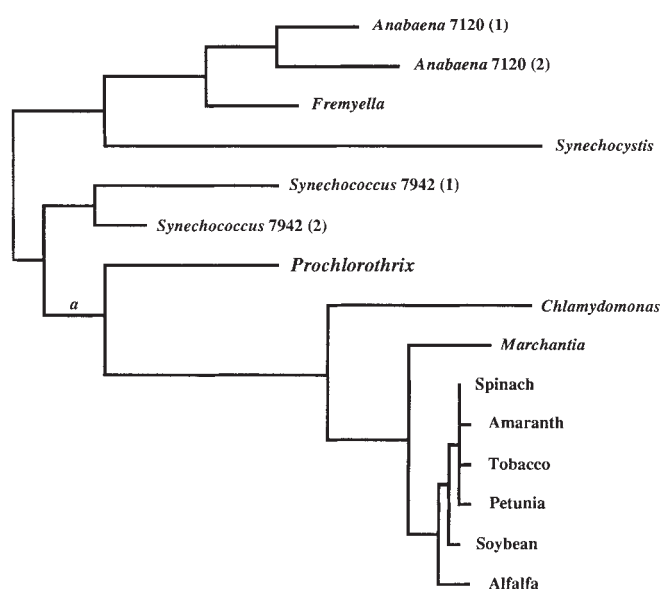


Fig. 3 Phylogenetic tree based on parsimony¹⁰ among amino-acid sequences derived from *psbA* gene sequences of thirteen species. Branch length reflects the number of character state differences unique to that branch and the amount of divergence from a most recent common ancestor, but is not meant to depict evolutionary time. The branch labelled *a* was found to be variable in length among the four different analyses (see text). Gene designations for *Anabaena* sp. strain PCC 7120 and *Synechococcus* sp. strain PCC 7942 are as in Fig. 2. Sequences identical to that of spinach and not shown are *Nicotiana debneyi*¹⁹ and *Solanum nigrum*²⁵. Data for sequences were obtained from the same data set as in Fig. 2.

Methods. 118 positions along the amino-acid sequences were variable for at least one of the 15 sequences analysed. Amino acids were converted to a numeric character for each of the variable positions. The following numbers of equally parsimonious trees were generated for each of the four treatments used to compare sequences: (1) 30 trees, (2) 15 trees, (3) 15 trees, and (4) 15 trees. The order of branching was the same in all trees for treatments 2–4 and in half of the trees generated using treatment 1 (see text), with some minor differences in the branching order of higher plants. Amino acids 348–354 at the C terminus of cyanobacterial sequences were excluded from the analysis which produced this tree. All data were considered unordered and the tree was produced rooted at the midpoint of the greatest patristic distance using the branch-and-bound option of PAUP¹⁰.

seems to be more chloroplast-like than cyanobacteria, *P. hollandica* may serve as a useful model organism for the study of oxygenic photosynthetic mechanisms.

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Bacterial growth blocked by a synthetic peptide based on the structure of a human proteinase inhibitor

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Cysteine proteinases are important not only in the intracellular catabolism of peptides and proteins¹ and in the processing of prohormones and proenzymes^{2,3}, but also in the penetration of normal human tissue by malignant cells⁴ and possibly microorganisms⁵, including viruses. Cystatin C is a human cysteine proteinase inhibitor present in extracellular fluids⁶. We have synthesized peptide derivatives mimicking the proposed proteinase-binding centre of cystatin C⁷ and find that they irreversibly inhibit cysteine proteinases. Several bacteria produce proteinases, so we tested a tripeptide derivative (Z-LVG-CHN₂) for *in vitro* anti-bacterial activity against a large number of bacterial strains belonging to thirteen different species. It was found to inhibit specifically the growth of all strains of group A streptococci. The susceptibility of these human pathogens to the peptide was compared with that to well-established anti-streptococcal antibiotics such as tetracycline and bacitracin. Moreover, the peptide was active *in vivo* against group A streptococci: mice injected with lethal doses of these bacteria were cured by a single injection of Z-LVG-CHN₂. The cysteine proteinase produced by group A streptococci was isolated and found to be inhibited by Z-LVG-CHN₂; moreover, excess proteinase relieved the growth inhibition caused by the peptide derivative, suggesting that the antibacterial activity of Z-LVG-CHN₂ is due to inhibition of this cysteine proteinase. This strategy of blocking proteinases with peptide derivatives that mimic naturally occurring inhibitors could be useful in the construction of new agents against other microorganisms, including viruses.

Of the peptide derivatives we synthesized to mimic the inhibitory reactive site of cystatin C (ref. 7), *N*-benzyloxycarbonyl-leucyl-valyl-glycine diazomethyl ketone (Z-LVG-CHN₂) most effectively inhibited papain, a model cysteine proteinase (association rate constant, $k_{+2} = 600,000 \text{ mol}^{-1} \text{ s}^{-1}$). The peptidyl portion of Z-LVG-CHN₂ was acting as a supersubstrate for the inhibited cysteine proteinase; the diazomethyl ketone group established a covalent link with the cysteine residue in the active site of cysteine proteinases⁸, thereby making Z-LVG-CHN₂ an irreversible inhibitor.

Many microorganisms produce proteinases, so we investigated the effect of Z-LVG-CHN₂ on bacterial growth. Paper

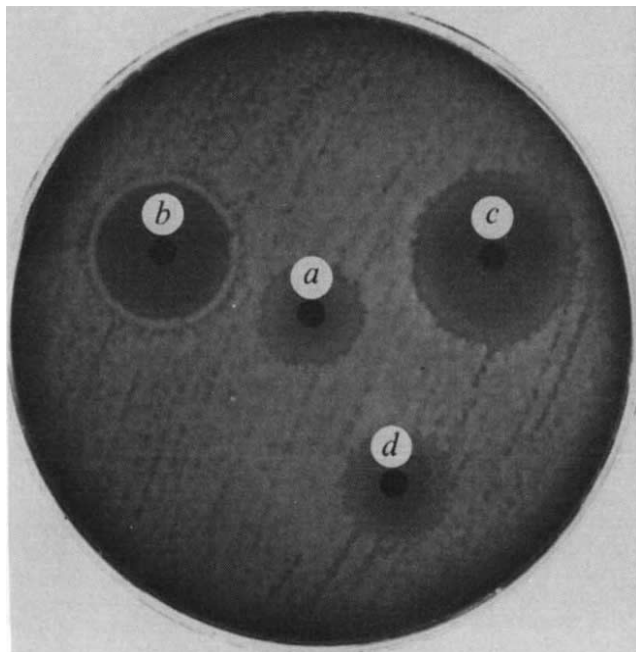


Fig. 1 Anti-bacterial activity of Z-LVG-CHN₂ in disc diffusion analysis.

Methods. Streptococci (strain 40/58 from the WHO Collaborating Center for Reference and Research on Streptococci, Institute of Hygiene and Epidemiology, Czechoslovakia) were plated on 0.8% purified agar in Todd-Hewitt broth containing 4% sheep's blood, and antibiotic-sensitivity discs were applied to the plate. In *a*, a non-impregnated disc had been prepared to contain 24 µg Z-LVG-CHN₂ by impregnating a sterile filter paper disc with 60 µl Z-LVG-CHN₂ (0.4 mg ml⁻¹) in 1% dimethyl sulphoxide in water. Disc *b* contained 30 µg doxycycline; disc *c*, 0.1 µg benzylpenicillin; disc *d*, 0.2 IU of bacitracin. All discs were from AB Biodisc, Sweden. Z-LVG-CHN₂ discs (24 µg per disc) were also used to screen a total of 190 strains of human pathogenic bacteria. All strains tested were from routine clinical specimens (Clinical Microbiology Laboratory, University Hospital in Lund). Species and number of strains were: *Staphylococcus aureus*, 12; *Staphylococcus epidermidis*, 14; group A streptococci, 28; group B streptococci, 14; group C streptococci, 19; group G streptococci, 11; *Streptococcus pneumoniae*, 8; *Streptococcus faecalis*, 10; *Branhamella catarrhalis*, 9; *Haemophilus influenzae*, 17; *Escherichia coli*, 22; *Klebsiella pneumoniae*, 15 and *Pseudomonas aeruginosa*, 11. All group A streptococcal strains showed a zone similar to *a* in the figure, with no bacterial growth surrounding the disc, whereas the other strains grew up to the edge of the Z-LVG-CHN₂-containing disc. In control experiments eight of the group A streptococcal strains were also tested against discs impregnated with 60 µl 1% dimethylsulphoxide or with 24 µg commercial (Sigma) cysteine proteinase inhibitor L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guandino) butane (E-64). No inhibitory activity was shown by either the dimethylsulphoxide solution or by E-64.

discs were impregnated with the synthetic inhibitor and applied to plates streaked with different bacteria (see legend to Fig. 1). Growth of all group A streptococcal strains tested was inhibited to a degree comparable with that shown by some well established antibiotics (Fig. 1); all other strains were unaffected by Z-LVG-CHN₂. We then analysed the anti-streptococcal activity of Z-LVG-CHN₂ in infected mice using a strain of group A streptococcus virulent in mice (strain 40/58). Ten outbred NMRI mice (ALAB, Sweden) were injected intraperitoneally (i.p.) with 10⁶ streptococci in Todd-Hewitt broth. Thirty minutes later we injected (i.p.) five animals with 0.2 mg Z-LVG-CHN₂ in 0.5 ml 0.15 M NaCl in 1% dimethyl sulphoxide and five with 0.5 ml 0.15 M NaCl in 1% DMSO without Z-LVG-CHN₂. All mice in the control group died within 24 h, whereas among those treated with Z-LVG-CHN₂, one mouse died after three days and the

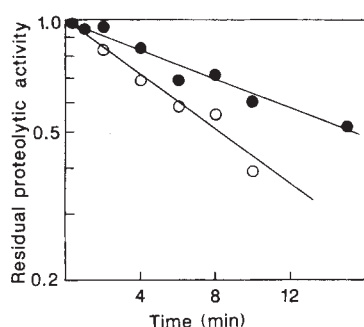


Fig. 2 Time-dependent inactivation of isolated streptococcal proteinase by 10 μM (●) or 20 μM (○) Z-LVG-CHN₂.

Methods. The streptococcal proteinase was isolated from growth medium of the streptococcal group A strain 40/58 from the Prague collection, as previously described⁸. The isolated enzyme was activated by preincubation at 25 °C during 1 h in 0.2 M sodium acetate/10 μM EDTA/50 μM dithiothreitol, pH 5.5. The activated enzyme (7.5 $\mu\text{g ml}^{-1}$) and Z-LVG-CHN₂ (from a stock solution in 1% dimethylsulphoxide) were mixed in the activation buffer at zero time. Samples were taken out at timed intervals and immediately added to assay tubes containing the chromogenic substrate Z-Lys-ONp (*N*- α -carboxybenzoyl-L-lysine-*para*-nitrophenyl ester HCl (Bachem) at a final concentration of 0.2 mM. After 20 min at 25 °C, the reaction was stopped by addition of buffered chloroacetate, and the absorbance was read at 340 nm. A time-dependent inactivation of the enzyme was found. The residual proteolytic activity in the samples as a function of time gave a linear semi-logarithmic plot typical of an irreversible second-order reaction. The rate of inactivation ($t_{0.5}$) at different Z-LVG-CHN₂ concentrations (10 μM and 20 μM) was dependent on the concentration of Z-LVG-CHN₂ ($[I]$). The apparent rate constant for association between enzyme and inhibitor, k'_{+2} , was then calculated as $\ln 2/t_{0.5}[I]$ (ref. 10).

remainder were still alive after two months. In a repeat experiment, all five control animals died within 24 h and the five mice treated with Z-LVG-CHN₂ survived (four weeks observation time). We then investigated the toxicity of Z-LVG-CHN₂: no ill-effects were seen in four mice each injected i.p. with 2 mg in 5 ml 0.15 M NaCl in 1% dimethylsulphoxide (two months observation time). So Z-LVG-CHN₂ is atoxic even at doses tenfold higher than those necessary to cure lethal streptococcal infections in mice.

Although Z-LVG-CHN₂ could inhibit streptococcal growth both *in vivo* and *in vitro*, the molecular mechanism behind this activity was not clear. There is a cysteine proteinase that is produced by all group A streptococcal strains but which has not been identified in other bacterial species, including other species of streptococci; the enzyme is produced as a zymogen that can be transformed into an active proteinase by reduction⁸. We purified the zymogen to apparent homogeneity from growth medium containing the same group A strain as we used in the animal experiments, when we found that the activated enzyme was irreversibly inhibited by Z-LVG-CHN₂. The association rate constant (k'_{+2}) of the reaction was determined as 102 $\text{mol}^{-1} \text{s}^{-1}$ (Fig. 2). Moreover, Z-LVG-CHN₂-mediated growth inhibition could be impeded by addition of the activated proteinase. These data indicate that Z-LVG-CHN₂ could be inhibiting growth of group A streptococci by blocking their cysteine proteinase. E-64 is a well-known cysteine proteinase inhibitor¹⁰, whose activity against the isolated streptococcal enzyme is comparable with that of Z-LVG-CHN₂, but E-64 did not inhibit growth of group A streptococci. This might be explained by the relatively greater hydrophobicity of Z-LVG-CHN₂ facilitating penetration of the bacterial cell wall, or by the fact that Z-LVG-CHN₂ is recognized by a permease, whereas E-64 is not. On this basis, proteinase-inhibiting peptide derivatives could be made more useful for antimicrobial therapy if their N-termini were recognizable by permeases that permit efficient intracellular uptake¹¹.

The biological function of the streptococcal proteinase is not known, but our data indicate that blocking the enzyme inhibits growth of these bacteria both *in vivo* and *in vitro*, which suggests that the proteinase is essential for the multiplication of group A streptococci. This is supported by the fact that we have so far been unable to isolate any mutants of group A streptococci devoid of the cysteine proteinase, neither have we seen any Z-LVG-CHN₂-resistant mutants of the 40/58 strain (frequency less than 10^{-6}). It will be interesting to see which of the other microorganisms that are known to require proteinases for multiplication and/or tissue penetration are affected by inhibitors that imitate supersubstrate sections of naturally occurring proteinase inhibitors.

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Cationic liposome-mediated transfection

P.L. Felgner and G.M. Ringold

A lipid named DOTMA has been created that forms unilamellar liposomes which complex with DNA and RNA for the transfection of mammalian cells, including suspension cells and hybridomas.

EFFORTS to develop methods for functionally delivering polynucleotides into living cells have continued steadily over the past several years. Effective methods include the use of calcium phosphate, liposomes, retroviral vectors, reconstituted viral envelopes and electroporation. In addition, there are several procedures which employ polycations such as polylysine, DEAE-dextran and polyornithine¹⁻³.

Recently, a DNA delivery reagent was developed which consists of polycationic liposomes comprised of a positively charged lipid named DOTMA, for (N[1-(2,3-dioleoyloxy)propyl]-N,N,N-triethylammonium)³. The advantages of this reagent over other methods have been demonstrated for a variety of cell types, and include improvements in convenience, reproducibility, efficiency and efficacy.

The reagent is particularly notable for its ease of use. An aliquot of the aqueous reagent is simply added to the tissue culture experiment together with the DNA or RNA of interest. Although the level of observed expression is heavily dependent upon the cell line and expression vector, in the vast majority of cases, expression levels are at least comparable to — and frequently much greater than — those obtained with either the calcium phosphate or DEAE-dextran methods³.

Mechanism of action

The postulated mechanism of action for the liposome reagent, based on experimental evidence, is illustrated in Fig. 1. DOTMA forms liposomes in an aqueous environment alone or together with other phospholipids^{3,4}, and the active reagent consists of sonicated vesicles containing equal parts of DOTMA and dioleoylphosphatidylethanolamine in water. DNA/DOTMA complexes form spontaneously after mixing an aliquot of the reagent with an aqueous solution of DNA³, trapping nearly all of the polynucleotide in the complex interior.

The ratio of DOTMA to DNA is critical to the reagent's efficacy^{3,5,6}. Theoretically, the optimal ratio should be such that the amount of positive charge contributed by the DOTMA liposomes slightly exceeds the number of negative charges on the DNA, so that the complexes have a net positive charge to facilitate their interaction with the negatively charged target cell surface. In practice, however, the optimum quantity of DOTMA is usually found to be roughly half this amount, and

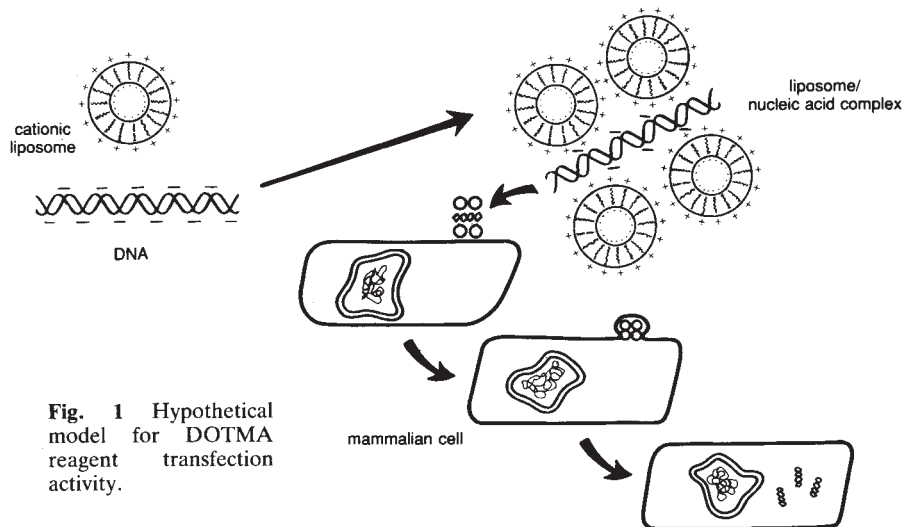


Fig. 1 Hypothetical model for DOTMA reagent transfection activity.

it is best to calibrate the concentration for each specific system to be studied.

We hypothesize that DNA/DOTMA complexes consist of four liposomes in association with each plasmid. Liposomes that are 250 nm in diameter — the approximate size of the vesicles in our reagent — contain about 2,500 lipid molecules⁷. Half of the molecules in each liposome, or 1,250 molecules, are positively charged DOTMA molecules. Because 5,000 negative charges are present in a standard 2,500-base-pair plasmid, approximately four liposomes are necessary to neutralize the negative charges of the plasmid DNA. The average diameter of the complexes is only slightly larger than that of the original liposomes as measured by laser light scattering (unpublished data), indicating that they contain a very small number of plasmids (perhaps only one) per complex. Complexes containing a larger number of plasmids would show a substantial, measurable size increase.

Liposomes comprised of DOTMA and dioleoylphosphatidylethanolamine fuse with negatively charged lipid membranes and with cell surfaces⁴. Micrographs of cells exposed to fluorescent DNA/liposome reagent complexes which contain rhodamine-labelled lipid also indicate cell-surface fusion³. Comparable fusion results obtained with a positively charged lipid analogue which is structurally very similar to DOTMA have recently been published⁸. Liposomes comprised of stearylamine or dioctyldecyl-dimethylammonium bromide were inactive in transfection assays (unpublished data).

Different subpopulations of cells and cell types seem to take up DNA/DOTMA

complexes with equal affinity. Following transfection with DNA/DOTMA complexes containing a fluorescent lipid analogue, essentially 100 per cent of cells become fluorescent³. Furthermore, cells exposed to radioactively labelled complexes adsorb 20-80 per cent of the added polynucleotide⁶. In a typical experiment in which 2 µg of a 2,500-base pair plasmid are exposed to one million cells (with approximately 10⁶ plasmids per cell) about 500,000 plasmids would be expected to be taken up per cell. But at the end of a two-day transfection experiment with mouse L- cell fibroblasts, only 300 intact psV2-CAT plasmids per cell were recovered in the nuclear fraction (unpublished data), indicating that less than one per cent of the adsorbed plasmids were reaching the nucleus intact. In a parallel experiment using the calcium phosphate method, however, less than 10 plasmid copies per cell were found in the nucleus.

In histochemical staining experiments on cells transfected with β-galactosidase expression vectors, up to 25 per cent of the cells were positive. It is not known whether the remaining 75 per cent of the cells were truly negative, or whether they were expressing activity below the sensitivity of the staining procedure: experiments with double-stranded RNA clearly show that more than 99 per cent of the cells are transfected⁵. Stable transformation efficiencies of as high as 10 per cent have been obtained, although this varies depending on vector and cell type.

The transfection step is performed in serum-free media to overcome the effects of an inhibitory component present in serum. The duration of this incubation

period ranges from 2 to 24 hours. Because purified sulphated proteoglycans are potent inhibitors of DOTMA-mediated transfection (unpublished results), a similar polyvalent negatively charged serum component is presumably responsible for this effect.

The efficiency of the method is essentially unaffected by variances in pH between 6.0 and 8.0³. Where a pH difference of as little as 0.1 in a calcium phosphate transfection experiment can influence the result, it has no effect on the DOTMA reagent¹. Precise mixing protocols, careful attention to the level of DNA, and the presence of low levels of contaminants are also less important to the outcome of DOTMA-mediated transfection experiments. In addition, large DNA molecules such as baculovirus DNA (130 kilobase pairs) have been successfully transfected into tissue culture cells using the technique (J. Barnett, personal communication).

Applications

Detailed quantitative data comparing the DOTMA reagent with other optimized transfection methods is available for only a few selected systems³, but some general statements regarding relative efficacy are possible. As with all transfection methods, the transient expression levels and transformation efficiencies obtained are strongly dependent upon the particular expression vector and the cell line to be tested. In a number of cell lines, 10- to 100-fold improvements over other methods have been demonstrated³. The reagent has also been used to transfect cells unresponsive to other methods (such as PC12 cells), suspension cells and hybridomas. The DOTMA liposome reagent has been used successfully to transfect, both stably and transiently, over 50 cell lines and primary cell cultures.

Relatively crude preparations of plasmid DNA, such as those made in mini-preps, can be efficiently introduced into cells using the reagent, allowing large numbers of different DNAs to be screened. For example, the products of site-directed mutagenesis can be conveniently transfected without time-consuming purification protocols.

Particularly exciting is the recent finding that the reagent is broadly applicable for the delivery and expression of messenger RNA in a wide variety of both adherent and suspension culture cell lines⁶. This has opened up a new experimental approach to the characterization of mRNA structure and function, areas that were not possible to study using more conventional transfection procedures. For example, the method has shown that the expression efficiency of mRNA synthesized *in vitro* is strongly dependent upon the presence of specific untranslated elements in the RNA construct⁶. The

Cell-to-cell communication

Products for examining the intricacies of cell function will be on hand next week when the American Society for Cell Biology and the American Society for Biochemistry and Molecular Biology hold their joint meeting in San Francisco, California. Below are several examples.

PROMEGA now offers confidential **cDNA and genomic library construction** services (Reader Service No. 101). The company will take polyadenylated RNA or genomic DNA, and construct a library using their line of vectors, including those prepared from lambda gt10, lambda gt11 and lambda gt11 *Sfi-Not*. These vectors allow cloning strategies such as directional cloning, subtraction library construction, and expression of cloned inserts as polypeptides fused with β -galactosidase. Each lambda library is packaged *in vitro* using Promega's Packagene extract, which the company says is prepared from a single strain devoid of *Eco K* activity to avoid destroying inserts with that restriction site. Promega will also isolate RNA from tissue, screen libraries, prepare plasmids and phage DNA, and produce antisera on a contract basis.

Benchtop power

Flow has a Cell Raiser of a **benchtop bioreactor** that prevents sleepless nights of worry and tedious trips to the lab to check growth parameters (Reader Service No. 102). Flow's bioreactor of that name comes complete with its own data acquisition system, so researchers can keep an



Flow's Cell Raiser bioreactor.

eye on fermentation data from home or a separate office using a modem. The Cell Raiser has built-in controls for pH, dissolved oxygen, agitation and temperature. Using its diagnostic software, the bioreactor's controller continuously tests its control loops, including tests for sensor operation and control elements. If any control value deviates from parameters set by the user, it sets off visual and

reagent has also been used successfully to deliver Sinbus virion RNA or *in vitro*-transcribed infectious RNA, resulting in active virus particles (S. Schlesinger, personal communication).

Despite the advantages offered by the liposome reagent, other transfection protocols may be more effective for some applications. A notable example is the optimized calcium phosphate procedure of Okayama¹, which gives excellent results in some cell lines. Similarly, for many suspension cultures, electroporation continues to be the only effective method for introducing nucleic acids. Despite these observations, the DOTMA-mediated transfection procedure stands out as one that can be broadly, successfully and conveniently applied to a very wide variety of different cell types for both stable and transient expression, and which can be applied to other polynucleotides, including both double-stranded⁵ and single-stranded messenger RNA⁶. Future studies with reagents of this type should reveal even more efficacious delivery systems for the introduction of small oligonucleotides, very large DNA frag-

ments or even proteins into intact cells.

The DOTMA reagent is now commercially available from Bethesda Research Laboratories (Bethesda, Maryland, USA) under the name Lipofectin. Still other cationic liposome delivery systems may eventually prove applicable to the delivery of polynucleotides *in vivo* for therapeutic applications. □

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audible alarms. The computer can also be set to call users remotely via a modem to deliver alarm information. What's better, users can alter parameter settings through the modem to fix minor problems.

Jouan has a **midrange benchtop centrifuge** that can be used to spin microtubes and bottles — without changing the rotor (*Reader Service No. 103*). Jouan's model C312 centrifuge comes with rotors equipped with plastic insert modules configured for labware ranging from 0.5 ml tubes to 250 ml bottles. The modules



Jouan's benchtop centrifuge comes with modular plastic tube inserts.

allow several types of samples to be centrifuged in one run, in different wells of the same rotor. The microtube inserts can also be used as a tube rack, so that experiments can be whisked from the benchtop directly to the centrifuge. The centrifuge's horizontal T4 rotor achieves up to 3,600 r.p.m., generating 2,550 g. The fixed angle rotor generates up to 6,300 g. The C312 centrifuge comes in both a non-refrigerated version and a refrigerated version which has a temperature range of 0–30 °C.

Yamato has incorporated the innovative technology of molecular sieving into a **freeze dryer** that fits onto the lab bench (*Reader Service No. 104*). Yamato has altogether eliminated bulky cooling mechanisms in its model DC41 freeze dryer: the unit silently "cages" sublimating water vapour molecules without dry

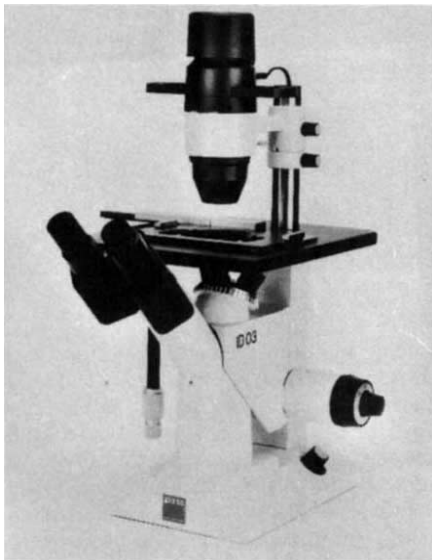


Yamato's bath-free freeze dryer.

ice, chilled alcohol baths or mechanical refrigeration. Heating the sieves releases the accumulated water and regenerates them for reuse. The freeze dryer has five ports, and can process 200 ml of sample at one time. Its instrument panel has a timer with a buzzer to signal the conclusion of the sublimation, and either a thermocouple vacuum gauge or a Bourdon Tube for measuring pressure in centimetres of mercury. The company says its freeze dryer is particularly useful for the preservation of temperature-sensitive biologicals.

The small world

Zeiss has rounded out its line of **inverted microscopes** with its low-priced model ID03 (*Reader Service No. 105*). The inverted microscope features a new 6 V, 20 W illuminator with a diffusing disk that Zeiss says assures even illumination in brightfield techniques. It contains an aperture iris diaphragm and a three-position slider that allows it to be switched instantly from brightfield to either of two phase-contrast techniques. The new illuminator in the model ID03 has a working



Zeiss's basic model ID03 inverted microscope comes with a host of features.

distance of 70 mm at 0.3 numerical aperture, and its housing has a flip-top for replacing the bulb.

Olympus's new SZH model **zoom stereomicroscopes** have a remote control facility for hands-free operation (*Reader Service No. 106*). Focusing is controlled using a foot pedal — a feature that Olympus says is particularly useful when dissecting using micromanipulation tools. Firm foot pressure is used to make rapid movements, and fine focusing is controlled by pressing gently. An anti-crash sensor prevents damage to the specimen or microscope. Olympus SZH stereomicroscopes have an 8.5:1 zoom ratio and a potential magnification range of 3.75 × to 384 ×. The microscopes can also be

equipped with remote control pedals for zoom function.

The Microvid **computer microscopy module** from Wild Leitz turns microscope eyepieces into a computer screen, so that data can be reflected into the microscope and photographed together with the image (*Reader Service No. 107*). The Microvid projects text, graphics and

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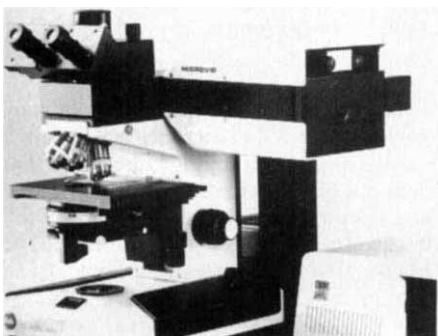
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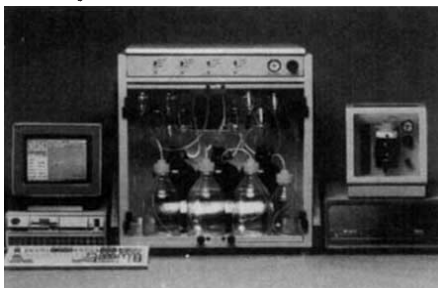


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DNA details

MilliGen/Biosearch, a division of Millipore Corporation, has announced the introduction of its model 8800 **large-scale DNA synthesizer** to meet the demand for



Large-scale DNA synthesis using β -cyanoethyl phosphoramidite chemistry.

large quantities of DNA in diagnostic and therapeutic applications (*Reader Service No. 108*). The model 8800 is a modular, benchtop instrument controlled by an IBM PC workstation which MilliGen/Biosearch says can synthesize from 30 to 300 μ mol of DNA at one time. The system has a batch reaction design which relies on base-dependent coupling and deblocking protocols. The fully programmable synthesizer has software which performs reagent calculations and has notebook capabilities. The standard protocols are based on β -cyanoethyl phosphoramidite chemistry, but the system can be adapted for other chemistries as well.

DNASar, Inc. has a **compact disk** containing the Brookhaven Library of Protein Structures which allows researchers to view the structures of protein molecules in three dimensions by wearing stereo glasses (*Reader Service No. 109*).

The company developed the X★RAY disk as a less expensive adjunct to high-performance molecular workstations for protein chemists, and as an aid for students wishing to familiarize themselves with 3-dimensional protein structures. DNASar says X★RAY rapidly paints the detailed composite molecular structures in colour stereo views on the screen of any IBM PC/AT/XT or PS/2 equipped with an EGA or VGA colour graphics monitor. The displayed molecules can be fully rotated with and without side chains, and can be printed out on a dot matrix or laser printer, or an HP Plotter.

Biotix, Inc. is introducing a **high-speed DNA synthesizer** at the San Francisco exhibition that it says is capable of pro-



Five-minute oligonucleotides with 99 per cent purity from Biotix.

ducing high-purity oligonucleotides with 99 per cent efficiency in coupling cycles of under five minutes (*Reader Service No. 110*). Both the one- and two-column models of the synthesizer are fully automated and user programmable. The synthesizer has a 200-mer capacity, and Biotix guarantees that it will synthesize oligonucleotides of up to 125 bases. The \$14,950 single-column instrument comes complete with an IBM-compatible PC.

Perkin-Elmer Cetus is now producing **recombinant Taq DNA polymerase** for use in the polymerase chain reaction (PCR) for DNA amplification (*Reader Service No. 111*). The company's AmpliTaq



Recombinant Taq DNA polymerase from Perkin-Elmer Cetus comes singly or as a kit.

DNA polymerase is genetically engineered and purified using an enzyme process that Perkin-Elmer Cetus says removes unwanted thermostable nucleases. The company says further that studies comparing AmpliTaq with native Taq DNA polymerase show that the thermostability and specificity of the recombinant enzyme is equivalent to that of the native form in the PCR process. AmpliTaq DNA polymerase is available separately in 250-unit, 0.05 ml microtubes, or as part of Perkin-Elmer Cetus' GeneAmp DNA amplification reagent kit. The company says researchers who are currently using native Taq DNA polymerase can switch to AmpliTaq without modifying their protocols.

Gels and more

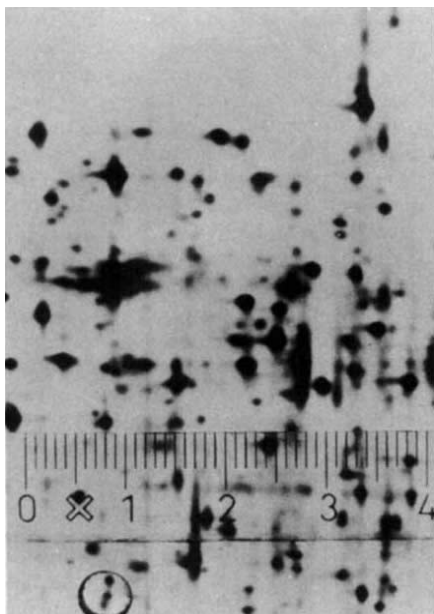
The new model 270A **analytical capillary electrophoresis** system from Applied Biosystems couples the separation power of electrophoresis with microprocessor-controlled instrumentation (*Reader Service No. 112*). The company says the system makes the fast analysis of a wide variety of molecules routine and minimizes the pre-run preparation time traditionally required by standard electrophoretic protocols. Unlike traditional gel methods, the model 270A automates the electrophoretic separation of molecules in free solution within small-bore capillary tubes, without a gel matrix or denaturant. The entire procedure is automated by an integral, menu-driven system controller.



Applied Biosystems brings analytical capillary electrophoresis to the lab with the model 270A.

Sample loss is virtually eliminated due to the small amount of sample required for analysis, says ABI, and the overall reagent and buffer consumption is extremely low because a small-bore capillary is used.

Bio-Rad has recently released an updated version of its Protean II multi-cell **two-dimensional gel electrophoresis** apparatus (*Reader Service No. 113*). The improved system comes with a more efficient cooling system that permits higher power settings with minimal diffusion. The gels are first attached to the three central cooling cores of the multi-cell, for uniform heat removal. Two



Results obtained using Bio-Rad's Protean II multi-cell 2D gel electrophoresis setup.

aluminium cooling coils coated with an electrically non-conductive material that exhibits high thermal conductivity are then placed in the lower buffer chamber to chill the second dimension running buffer. The gels are run submerged in the chilled running buffer for optimal heat dissipation. Two new detergents that eliminate equilibration and overnight focusing steps — CHAPS and NONIDET P-40 — are available for use with the system, which comes complete with a step-by-step protocol manual.

Amersham International now offers its Hyperfilm-MP and Hyperfilm- β max high-performance autoradiography films in rolls of 20 cm $\times$ 25 m (*Reader Service No. 114*). The larger films are designed for applications which require longer pieces of film than the sizes currently available in sheets, such as DNA sequencing, where users are frequently forced to splice two pieces of film together. For applications such as whole body autoradiography and thin-layer chromatography which require less than one conventional sheet of film, the rolls are more economical. Amersham's Hyperfilm- β max roll film is intended for high-speed, high-resolution direct autoradiography of compounds labelled with ^{14}C , ^{35}S or ^{125}I . The Hyperfilm-MP film is for use with ^3H , ^{14}C , ^{32}P or ^{125}I , and can be used with intensifying screens or fluorography.

Molecular Dynamics says its new HRDI Autophoresis 1000A instrument will do for electrophoresis what the telescope did for astronomy (*Reader Service No. 115*). The company's astronomical claims centre on the high-resolution dynamic imaging (HRDI) properties of the instrument, that allow the detection

of bands in electrophoresis gels in real time, without staining. The Autophoresis system not only eliminates gel staining steps and autoradiography, but also allows the user to determine when the compounds of interest are sufficiently separated. Because diffusion is virtually eliminated, Molecular Dynamics says smaller compounds — such as peptides and other molecules which either diffuse rapidly or do not stain well — can be detected. The HRDI optical technique is related to the phase contrast imaging used in microscopy. The gel is illuminated by a HeNe laser light source which projects the image of the gel onto a screen. Portions of the gel which do not contain sample bands appear as dark areas on the screen because light which passes straight through the gel is absorbed by a mask. Bands in the sample bend the light so that it bypasses the mask and is focused on the viewing screen.

Separation and analysis

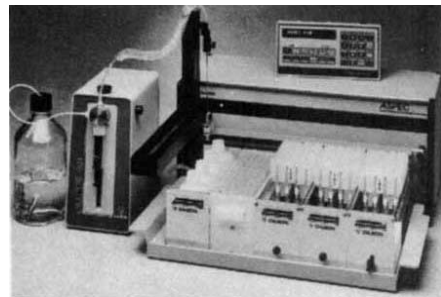
The GPC System for gel permeation chromatography from Spectra-Physics



Spectra-Physics offers a gel permeation chromatography system for less than \$14,000.

includes modular LC hardware, comprehensive software and a selection of columns (*Reader Service No. 116*). The system consists of two parts: the chromatography hardware including a pump, detector and column oven which the company says is capable of delivering performance repeatability in the one per cent range; and the multi-tasking software which runs on either a Spectra-Physics WINner+ or a ChromStation/2 workstation/integrator. The GPC software performs standard operations, collects and stores raw data, calculates molecular weight distribution, plots raw chromatograms, and calibrates using narrow range, broad range or universal standards. Time/area slices or complete reports can be stored automatically, and reports can be customized to fit specific requirements. Once the data are stored, annotated replots of original chromatograms can be produced with drawn-in baseline. The complete hardware system costs \$11,300 (US) and the accompanying software, \$1,250 (US).

Gilson Medical Electronics has a sample preparation system that both cleans a sample automatically and injects the prepared sample into an on-line HPLC system without supervision — the



Busy labs will benefit from Gilson Medical Electronics' ASPEC system.

ASPEC system (*Reader Service No. 117*). Gilson says the ASPEC system is compatible with most standard 100-mg and 500-mg disposable extraction columns and can process up to 108 complex samples. ASPEC can be used automatically to perform extraction, elution and concentration steps, or for liquid handling operations such as transfers and dilutions. The software is flexible so that operators may select the proper volume and flow rate of each solvent at each extraction step, or program a unique operational sequence. Applications of the ASPEC system include liquid-solid extractions of hormones and drugs in biological fluids, nucleic acids and lipids in tissue/cell extracts, and pesticides in soil and water.

The model 338 gradient liquid chromatograph from Beckman Instruments links reliable pumps with programmable UV-visible detectors and provides single-point instrument control (*Reader Service No. 118*). The system's model 110B pump flow rate range of 0.01–10 ml min⁻¹ can be increased to 28 ml min⁻¹ with the addition of preparative pumpheads, so that only one system is needed for analytical, high-

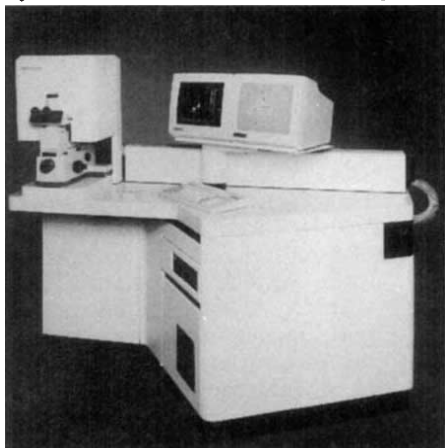


Beckman's model 338 gradient liquid chromatograph.

speed and semipreparative chromatography. The variable wavelength Beckman UV-visible 166 detector offers sensitivity down to 0.001 AUFS with low refractive index response and stable baselines, says the company. Results can be optimized automatically through the built-in time programming of absorbance range, wavelength and auto zero. The model 338 system's controller is a NEC lap-top computer, which is linked through an analogue interface to the pump and digitally to the detector module. For more powerful data handling, the system can be used with a desktop PC running Beckman's System Gold software.

A look inside

Meridian Instruments has recently introduced its ACAS 570 **interactive laser cytometer** for adherent cell sorting and



A full range of laser cell analyses are possible using Meridian's ACAS 570.

quantitative fluorescence analysis (Reader Service No. 119). A focused laser spot provides the capability to use interactive methods, as well as to perform automated quantitative fluorescence imaging and analysis, confocal microscopy, and the measurement of intracellular ions such as calcium and pH. Meridian's instrument can be used for optical sectioning, intercellular communication studies, the real-time measurement of cellular dynamics, and the isolation of mutant or transfected cells for propagation or analysis using a unique patented process called "cookie cutter". The ACAS 570 uses an argon ion laser and dual photometric detection system for single or simultaneous dual parameter or ratio measurements on single cells or entire cell populations. Meridian says the system provides optimal sensitivity and quantitation of fluorescence measurements with minimal photobleaching.

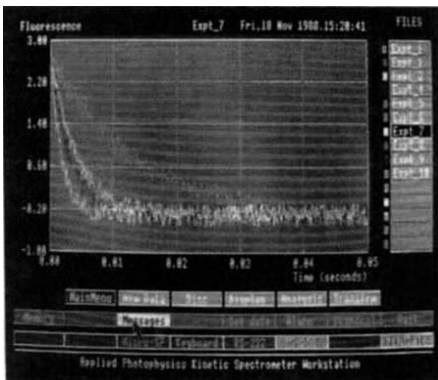
Hitachi Instruments has a new **intracellular calcium measurement system** designed for use with Quin, Fura or Indo dyes — the model F-2000 (Reader Service No. 120). A sample introduction system combined with report generation for both tabulated and graphical data provide a



Quin, Fura or Indo dyes can be used with Hitachi's new calcium measurement system.

complete package for the application. The F-2000 features a wavelength slew rate of 200 nm s⁻¹ so that a maximum number of data points can be measured during the analysis. Hitachi says the horizontal beam geometry provides a four-fold increase in sensitivity and allows samples of 0.6 ml to be measured routinely in a standard 1 cm cuvette.

Applied Photophysics will be launching two new **spectrometry instruments** at the San Francisco meeting (Reader Service No. 121). The company's model SF.17MV microvolume biocompatible stopped-flow spectrofluorimeter requires just 100 µl of each reactant to prime the sample flow circuit. Comprehensive absorption and fluorescence capabilities, facilities for anaerobic and large-ratio mixing, and a high-performance 32-bit RISC-processor spectrometer workstation



The screen display of Applied Photophysics's stopped-flow spectrofluorimeter.

are provided as standard. Applied Photophysics' model RX.1000 rapid mixing spectrometer accessory can be fitted to any absorbance or fluorescence spectrometer that has a regular 1-cm cuvette holder. The company says the accessory can reduce the shortest half-life measurable on a spectrometer from minutes down to less than one second. Designed to satisfy the users of precious and sensitive chemical and biological samples, the accessory also features an anaerobic capability and large-ratio mixing, and offers comprehensive thermostating facilities.

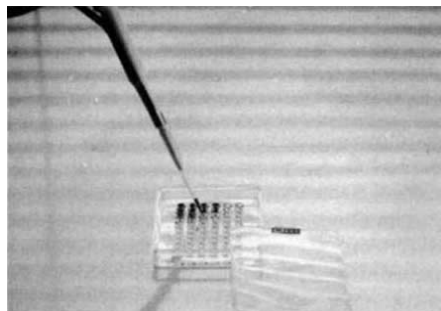
Enzymes, etc.

The radiochemicals division of ICN Biomedicals, Inc. has expanded its series of ¹²⁵I-labelled immunoglobulins with the addition of **goat anti-mouse IgM** ¹²⁵I (Reader Service No. 122). The antibodies are prepared by immunizing the appropriate host with a purified immunoglobulin from normal serum. The hosts are bled, and the antisera is pooled and then affinity-purified to ensure species specificity. The purified antibodies are then labelled by a mild chloramine-T method and further purified by HPLC. Each batch is tested by measuring its binding to the appropriate immunoglobulin.

Seikagaku America offers a host of products for **cell culture** and manipulation, including enzymes for mucopolysaccharide lysis, cell wall lysis, glycosidases, reverse transcriptase, two inhibitors of myosin light chain kinase and protein kinase activators (Reader Service No. 123). The company's Cellmatrix medium is a collagen gel for culturing cells and tissue. Seikagaku says Cellmatrix is superior to rat-tail collagen because it forms gels more rapidly and reproducibly yields higher strength gels. The medium is useful not only for coating tissue culture surfaces, but for collagen gel matrix culture. Cells can be grown inside the gels to mimic three-dimensional growth *in vivo*.

Pipettes and plates

Pharmacia LKB offers a **microplate for DNA sequencing** which will allow researchers to perform all of the sequencing reactions in a handy sample holder, with no need for tubes and racks (Reader Service No. 124). The company's micro-sample plate is specifically designed for Sanger reactions: each of its 60 wells accommodates 15 µl and reagents can be added directly to the mixture. The new DNA strand is separated by simply floating the plate on the surface of boiling



Pharmacia LKB's microsequencing plate goes from bench, to bath, to freezer.

water. No evaporation of sample is required. After the gels are loaded, the sample holder can be covered with the lid provided and placed in the freezer.

Eppendorf has a **pipette** for dispensing solutions directly from tall bottles (Reader Service No. 125). The company's Multipipette can now be coupled with its Combilong adaptor to reach into the depths of large bottles. The Combilong consists of an aspirating tube and a chamber which has to be filled with solution. The liquid level in the chamber remains constant: the Combipip of the Multipipette takes the liquid directly from the reservoir. The 30-cm long Combilong can be shortened to any length. □

These notes are compiled by Carol Ezzell from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.